ENERGETIC SIGNALS AND STRESSORS REGULATE REPRODUCTIVE-IMMUNE TRADE-OFFS AND SEASONAL SICKNESS RESPONSES IN SIBERIAN HAMSTERS

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Biology,
Indiana University
April 2015

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ACKNOWLEDGMENTS

This dissertation would not have been possible without the support of many individuals, groups, and funding sources. First, I would like to thank Dr. Gregory Demas for guiding me through this process. Greg gave me the perfect combination of independence and mentorship. He always listened to and valued my input and questions and often treated me more like a collaborator than a student. I am incredibly grateful for this relationship, as I feel I am finishing my graduate work as a confident and independent thinker. I thank my dissertation committee members, Drs. Noah Ashley, Laura Hurley, Ellen Ketterson, and Troy Smith for their feedback and guidance. I appreciate their enthusiasm for my ideas and their support of me outside of my research endeavors, as they exposed me to exciting ideas in their classes and provided opportunities for me to use my skills outside of the laboratory. I would also like to thank my undergraduate research mentors, Drs. Bob Mauck and Mark Haussmann. They were influential in getting me to think like a scientist, and I am so grateful that they took me on as their student during my sophomore year. This dissertation research would have not been possible without funding from the National Science Foundation (a NSF Graduate Research Fellowship and a NSF Doctoral Dissertation Improvement Grant), the National Institutes of Health (a T32 Common Themes in Reproductive Diversity training grant), and Sigma Xi (Grant-in-Aid of Research).

The “Demas Lab Ladies” were a huge part of my success in graduate school. I am so thankful that Emily Chester took me under her wing from my first day in the lab until she graduated. She taught me how to do many of the techniques that I

utilized in this dissertation. Nikki Rendon and Allison Bailey were with me through every step of this process. They helped me with many of these experiments and were there to provide advice whenever I needed it. These ladies became two of my greatest friends, and I have no doubt that we will remain lifelong friends. Our newest labmate, Kristyn Sylvia, was a great help throughout my final two years of graduate school. She is a wonderful addition to the lab, and I very much appreciated her spunk and sass these past two years. Additionally, I have had the pleasure to work with some remarkable undergraduates while in the lab---especially Candace Cooper and Chryssa Athans. They were not only incredibly talented and hard-working, but they were a joy to mentor. Finally, thank you to the Jordan Hall animal care staff for providing exceptional care for our hamsters.

Throughout this process I was fortunate to the have the support of many people outside the lab. My parents, Jeff and Lynne, and my brother, Charlie, have been some of my greatest cheerleaders. They've talked me through all the ups and downs, and I'm so glad they were always just a phone call away. I am very appreciative of the amazing friends I have made throughout this process---I am especially grateful to CJ Jewell, Rebecca Penny, Leticia Mayberry Wright, and Sarah Zukowski for keeping me occupied outside of the lab and for being willing to listen to my thoughts and provide advice.

Lastly, I am ever so thankful to Dan Lithio for his love and encouragement. From bringing me dinner in the lab to becoming BIACUC certified to help me with my experiments, he truly has been a supporter of all my endeavors.

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ENERGETIC SIGNALS AND STRESSORS REGULATE REPRODUCTIVE IMMUNE TRADE-OFFS AND SEASONAL SICKNESS RESPONSES IN SIBERIAN HAMSTERS

To ensure survival and reproductive success, animals must optimally allocate energy among various physiological and behavioral processes while inhabiting environments that change predictably across and unpredictably within seasons. In this dissertation, I examined the mechanisms by which a seasonally-breeding rodent allocates energy between the reproductive and immune systems within the breeding season and modulates intensity of its sickness responses to a simulated infection across seasons. Siberian hamsters (*Phodopus sungorus*) inhibit reproduction and display lower body masses, lower levels of the adipose hormone leptin, and less intense sickness responses when housed in short, winter-like days compared to long, summer-like days. I used several techniques to modulate the internal energetic state of the hamsters (i.e., food restriction, manipulation of the energetic hormones leptin and insulin, pharmacological induction of glucose deprivation) to determine its role in reproductive-immune trade-offs and seasonal regulation of sickness intensity. In the first chapter, I examined energetic mechanisms involved in regulating reproductive-immune trade-offs in reproductively-active female hamsters. I found that glucose deprivation resulted in reproductive suppression, however, suppression could be alleviated when animals were provided with a hormonal signal of increased fat stores (i.e., leptin). Alternatively, reproduction was not inhibited when animals experienced more

severe glucose deprivation; yet, providing animals with the signal of increased fat stores during this period of severe glucose deprivation resulted in decreased allocation to humoral immunity. In the last three chapters, I examined the contributions of seasonal changes in energetic fuels and signals to seasonal variation in sickness intensity. I found that seasonal variation in sickness-induced hypothermia was regulated by seasonal changes in glucose availability and leptin levels; however, seasonal changes in sickness-induced anorexia and body mass loss were regulated by seasonal differences in body mass more generally. Finally, I observed that changes in insulin, a pancreatic hormone secreted in response to positive energy balance, had both suppressive and enhancing effects on sickness intensity depending on energetic context. These collective findings illustrate that physiological trade-offs and sickness intensity are sensitive to a variety of energetic modulators and that the effects of these modulators are dependent on their interactions with each other and the environment.

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INTRODUCTION

Excerpts included from: Carlton, E.D., Demas, G.E., French, S.S., 2012. Leptin, a neuroendocrine mediator of immune responses, inflammation, and sickness behaviors. *Hormones and Behavior* 62, 272-279.

Mounting an appropriate immune response to infection is fundamental to survival. Immunity is not only modulated in response to pathogen type and infection severity, but the strength of an immune response can be influenced by demands from other physiological systems and changes in environmental conditions. Animals have evolved mechanisms to maintain optimal immune function despite variations in their internal and external environments. As such, specific mechanisms facilitate energy allocation toward and away from immunity based upon an organism's current energetic needs and demands.

Energetic trade-offs

The majority of free-living animals have relatively finite energy stores that they must allocate to different biological processes, such as reproduction, growth, thermoregulation, and immune function (Sheldon and Verhulst, 1996). Immune responses are energetically costly to mount, and quantification of these costs has shown that inoculation with even a very mild antigen can increase oxygen consumption by approximately 20 percent (Demas et al., 1997a). In some cases, the energetic costs of mounting an immune response may be sufficiently great that allocation to biological processes other than immune function is compromised. For example, animals stimulated with an immune challenge while engaging in reproduction and rearing of offspring often show reduced reproductive success

(Bonneaud et al., 2003; Ilmonen et al., 2000; Uller et al., 2006). Similarly, animals that are mounting an immune response may show reduced growth and development (Fair et al., 1999; Romano et al., 2011).

The severity of these trade-offs is largely dependent on the quality of the animal's external environment, specifically the availability of energetic resources. Some of the strongest evidence that trade-offs among physiological processes are a result of limited energetic resources come from studies where food availability is manipulated within the laboratory (French et al., 2009b). For instance, female tree lizards that are yolking eggs (the most energetically expensive component of reproduction in this species) and provided with restricted access to food under laboratory conditions take twice as long to heal a wound as compared with non-reproductive females provided a similarly restricted diet. When food is provided *ad libitum*, however, there is no difference in the wound healing time between the reproductive and non-reproductive groups (French et al., 2007). Clearly, balancing these trade-offs among competing physiological systems via appropriate allocation of energetic resources can be critical for maximizing an animal's current health, survival, and reproductive success.

Seasonality

Outside of laboratory settings and in the wild, organisms face energetic trade-offs as a result of seasonal changes in their environment. Animals that inhabit temperate zones face challenges that are products of temporal changes in resource availability and climate fluctuations (i.e., changes in temperature, precipitation, humidity). These changes in resource availability and environmental conditions

occur across the annual cycle. For example, the summer months coincide with warm temperatures and increased food availability, which provide organisms with an opportune time to engage in the energetically costly behaviors associated with reproduction (e.g., courtship rituals and territory defense, gestation or egg incubation, and offspring care) (Bronson, 1985). However, during the winter months, low temperatures lead to reduced food availability and increased thermoregulatory demands, and allocating energetic reserves toward activities that are non-essential for survival is often not an option. These fluctuations in environmental resource availability occur in a mostly predictable manner, and as such, animals inhabiting these temperate zones have evolved mechanisms that allow them to meet the challenges that accompany these seasons.

The most predictable signal of the changing seasons is the change in day length (i.e., photoperiod) across the annual cycle. As the colder, winter months approach, the days become shorter, and as the warmer, spring months draw near, the days become longer. This environmental photoperiod signal can be translated into a physiological signal via release of the indoleamine hormone melatonin (Goldman, 2001). Melatonin is secreted by the pineal gland during the dark phase (i.e., night) and its secretion is blocked by exposure to light. It is the daily increases in the duration of melatonin release that provide an organism with a physiological cue of the approaching winter months and the decreases in the length of secretion that signal the approach of the spring and summer months. Therefore, by utilizing this stable photoperiodic cue, animals in non-tropical zones can time the onset and

termination of reproductive events to occur during the months where the climate is mild and food is most abundant.

Reproduction may be the most obvious and well-studied trait under photoperiodic control, but photoperiodic cues also coordinate seasonal changes in immune function (reviewed in Nelson, 2002). To combat threats to survival via exposure to pathogens in the winter, some animals show enhancement of their immune defenses. Specifically, several species of birds and small mammals show enhanced immunity when housed in laboratory conditions where they are exposed to short, winter-like day lengths as opposed to long, summer-like day lengths (Nelson and Demas, 1996).This short-day enhancement of immunity that is observed in the laboratory might result from immune system upregulation to counteract the immunosuppressive effects of winter environmental stressors like low temperatures and reduced food abundance, so that in the wild, summer and winter immune function does not differ. This relationship was supported in deer mice (*Peromyscus maniculatus*) as short-day enhancement of immune function disappeared when food was restricted and the lab temperature was lowered to simulate winter conditions (Demas and Nelson, 1998). Many of the investigations of seasonal variation in immune response have examined variation in immune parameters that require very little (i.e., constitutive immune defenses) to moderate (i.e., antigen-induced antibody production) energetic investment (Klasing, 2004). However, proper seasonal regulation of more expensive acute phase response (APR), and the associated sickness behaviors that accompany it, may be one of the

more critical immune responses to regulate to ensure survival and reproductive success in a temperate environment.

Sickness responses

A critical component of immunity, and one of the first responses of the body to infection, is the APR, and subsequent behavioral and physiological manifestations of sickness that accompanies it. During the APR, pro-inflammatory cytokines are released from activated neutrophils and macrophages and aid in the recruitment of additional immune cells to the local site of infection. These cytokines also act on the brain to generate sickness responses, which are characterized by additional release of pro-inflammatory cytokines, fever or hypothermia, anorexia, reductions in social, exploratory, and sexual behaviors, and hypothalamic-pituitary-adrenal (HPA) axis stimulation (Hart, 1988; Tizard, 2008). While behavioral patterns associated with sickness responses may appear to be a result of infection-induced weakness, these responses are actually part of a well-adapted mechanism to aid the host organism in clearance of the infectious agent (Hart, 1988). For instance, fever acts to inhibit the growth of some viral and bacterial pathogens, and the elevated temperature may increase the efficiency of immunological responses by enhancing bacterial killing by neutrophils and lymphocyte proliferation (Kluger, 1986). Additionally, blocking the fever response increases the likelihood of death in response to infection in lizards, fish, and rabbits, suggesting that the fever response is critical for animals to survive illness (Covert and Reynolds, 1977; Kluger et al., 1975; Vaughn et al., 1980). Anorexia may also be adaptive because it promotes a more efficient immune response (i.e., force-feeding some organisms

to achieve normal dietary intake during sickness results in increased mortality) or it may lead to greater stringency in diet selection, which allows an animal to alter its internal environment rendering it less favorable for pathogen growth (Kyriazakis et al., 1998; Murray and Murray, 1979; Tizard, 2008). Mounting a sickness response is clearly beneficial to an organism's survival, but being sick is also a significant energetic investment. Maintaining a fever results in an increased metabolic rate (Buchanan et al., 2003; Maier et al., 1994), while anorexic behavior limits an animal's energetic stores and contributes to body mass loss during illness.

Much of the work on sickness responses in this area has been performed by experimentally inducing sickness with lipopolysaccharide (LPS), an inert antigen from the cell wall of gram-negative bacteria. LPS is recognized by Toll-like receptor (TLR) 4, and activation of TLR4 stimulates the production of the pro-inflammatory cytokines, interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, which are released by activated neutrophils and macrophages and then act on the brain via the vagus nerve or humoral pathways. Ultimately, it is a combination of an animal's infection-induced release of pro-inflammatory cytokines, sensitivity to these pro-inflammatory cytokines, and the magnitude to which anti-inflammatory mechanisms suppress the effects of these pro-inflammatory cytokines that controls sickness response intensity (Konsman et al., 2002; Quan and Banks, 2007).

Seasonal variation in sickness responses

Studies of sickness responses in several seasonally breeding species have shown that the magnitude of an animal's response to infection varies across

seasons. For instance, in response to LPS, male Siberian hamsters (*Phodopus sungorus*) that are exposed to short days display shorter fever durations, less pronounced decreases in body mass and food intake, and reduced releases of the pro-inflammatory cytokines, IL-1β and IL-6 relative to long-day hamsters (Bilbo et al., 2002). Similarly, captive male Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) show greater weight loss and reduction in food intake in response to LPS injection when housed in long as compared to short days (Owen-Ashley et al., 2008; Owen-Ashley et al., 2006). In contrast, free-living male song sparrows (*Melospiza melodia morphna*) show more pronounced sickness behaviors in the winter, non-breeding season as compared to the spring, breeding season. For instance, in the winter, but not the spring, these males decrease territorial aggression and lose body mass in response to LPS injection (Owen-Ashley and Wingfield, 2006). Although seasonal breeders, captive male Puget's white-crowned sparrows (*Z. l. pugetensis*) show no differences in LPS-induced weight loss and food intake when housed in either long or short days (Owen-Ashley et al., 2008). Although these studies suggest that at least some seasonally breeding animals show variation in sickness intensity across the seasons, no clear patterns (i.e., summer vs. winter; breeding vs. non-breeding season) emerge at first glance. However, if the relationships between sickness and season are interpreted in regards to the seasonal changes in energetic condition of these organisms, one pattern becomes strikingly clear: sickness behaviors are attenuated in the season in which the organism has the lowest energy reserves.

Siberian hamsters and Gambel's sparrows have lower body masses and body

fat stores when housed in short day conditions compared to long day conditions(Bilbo et al., 2002; Owen-Ashley et al., 2006), and the free-living song sparrows that showed greater sickness intensity in the winter have greater body masses and fat in the winter months as compared to spring months (Owen-Ashley and Wingfield, 2006). While the Puget's sparrows do not show photoperiodic variation in sickness intensity while in captivity, they also do not show photoperiodic variation in body mass or fat stores in their captive environment (Owen-Ashley et al., 2008). Furthermore, individual variation in body mass is a strong predictor of individual variation in sickness response magnitude in Gambel's sparrows. There is a negative correlation between initial body mass and the percent decrease in body mass in response to LPS injection across all birds (short- and long-day housed), showing that initially fatter birds exhibit greater weight loss in response to LPS-induced sickness (Owen-Ashley et al., 2008; Owen-Ashley et al., 2006). In general, these observations suggest that the magnitude of a sickness response display (i.e., specifically those characteristics of the response that are energetically expensive) is constrained by a minimum body mass that the animal can reach before it risks not being able to recover and survive (Ashley and Wingfield, 2012; Owen-Ashley and Wingfield, 2007). This hypothesis has not yet been explicitly tested, but investigations of the endocrine mechanisms that contribute to variation in sickness intensity may shed light on how energy reserves may limit the expression of sickness responses in certain seasons.

Energetic fuels and their neuroendocrine mechanisms of immune system regulation

Over the past few decades, it has become increasingly clear that the immune system does not act in autonomy to clear infectious agents from an animal. Rather, it is the interplay among the immune, endocrine, and nervous systems that facilitates effective pathogen clearance. Communication among systems is therefore necessary to coordinate immune responses that are well suited to an organism's current physiological state, and in particular, the organism's nutritional condition. There is now little doubt that the development of the immune system and intensity of immune responses are constrained by access to and utilization of nutritional and energetic resources in an organism's environment (Faggioni et al., 2001; Kelly and Coutts, 2000; Lochmiller and Deerenberg, 2000; Long and Nanthakumar, 2004).

Glucose and insulin

Glucose is the primary source of energy for animals, as it fuels processes ranging from muscle movements to immunity to cognitive functioning (Mergenthaler et al., 2013; Wolowczuk et al., 2008). Glucose, itself, is not an endocrine molecule, however, changes in its levels within an organism results in activation of an endocrine cycle that facilitates its regulation. For instance, if blood glucose concentrations fall too low, the peptide hormone glucagon is released from the pancreas to stimulate conversion of stored glycogen into glucose; whereas high blood glucose concentrations result in the release of the pancreatic peptide hormone insulin which causes storage of excess glucose in the form of fat. Specifically,

glucose acts as a fuel for inactivated and activated lymphocytes and neutrophils, and (Calder, 1995), and as such, glucose availability and signals of glucose availability have the potential to modulate immune responses.

Experimental manipulations of glucose availability with 2-deoxy-D-glucose (2-DG), a non-metabolizable glucose analog that prevents cellular uptake of glucose and induces glucoprivation (Horton et al., 1973), have provided insight into the importance of glucose availability for immune responses. Reducing glucose availability via 2-DG decreases lymphocyte proliferation in response to stimulation with a mitogen, reduces leukocyte counts, and enhances pro-inflammatory cytokine production (Chou et al., 1996; Dreau et al., 1997; Lysle et al., 1988; Miller et al., 1994; Miller et al., 1993). Glucose availability also regulates immunity in a seasonal context. For instance, glucose deprivation results in decreases in splenocyte proliferation in long-day housed female deer mice, however, has no influence on splenocyte proliferation in short-day housed animals (Demas et al., 1997b). Similarly, glucose deprivation causes decreases in antibody production in long-day housed female Siberian hamsters, but does not change antibody production in short-day housed females (Zysling and Demas, 2007). These results suggest that glucose availability may be more critical for immune regulation when animals are reproductively-active. As such, inducing glucose deprivation decreases reproductive tissue masses in Siberian hamsters (Zysling and Demas, 2007) and impairs estrous cycling in Syrian hamsters (*Mesocricetus auratus*) (Schneider and Wade, 1989), and there is some evidence that glucose

deprivation may result in the expression of energetic trade-offs between the reproductive and immune systems in Siberian hamsters (Martin et al., 2008a).

Much of the work on the effects of insulin on immune responses has been performed to understand the connections between inflammatory responses and insulin resistance and for its potential use as a therapeutic treatment during sepsis (Dandona et al., 2004; Shoelson et al., 2006; van den Berghe et al., 2001). Few studies have examined the role of insulin as an energetic signal that may modulate of immune and sickness responses in healthy individuals. Insulin receptors are expressed on immune cells in both healthy and obese individuals (Bar et al., 1976; Krug et al., 1972), and in healthy individuals, insulin levels correlate with adipose tissue mass and change rapidly, yet still in proportion to the animal's general adiposity, in response to alterations in energy balance (Benoit et al., 2004). One study, however, has demonstrated that insulin may act as an energetic signal by which immunity is modulated in healthy individuals. Treating Siberian hamsters with exogenous insulin results in increased antibody production in short-day housed hamsters and a trend toward increased antibody production in long-day housed hamsters (Garcia et al., 2010), suggesting that insulin may act as an energetic modulator of immunity by facilitating enhanced immune responses.

Adipose tissue and leptin

Adipose tissue is composed of fat cells (i.e., adipocytes) which store fat and whose energy content can be liberated during negative energy balance. Adipose tissue is not only used for energy storage, but is an endocrine organ that secretes hormones and cytokines (Ahima and Flier, 2000). Reductions in adipose tissue in

non-obese animals can result in decreases in immune function (Demas et al., 2003; Demas and Sakaria, 2005), while inducing obesity can also cause immune impairments (Amar et al., 2007; Smith et al., 2007). Thus, adipose tissue and its endocrine actions appear to play critical roles in the regulation of immunity.

Leptin is one of the hormones that is secreted by white adipose tissue (WAT) and functions in immune system regulation. Leptin, a peptide hormone, acts as a potent anorexigenic agent, and circulating concentrations of the hormone are directly proportional to adipose tissue mass (Ahima and Flier, 2000; Maffei et al., 1995). High levels of leptin indicate adequate energy stores, whereas low circulating levels of leptin are consistent with an energy deficit and may direct the partitioning of energetic resources toward different systems. Leptin is categorized as a cytokine hormone, as its structure is similar to members of the type 1 cytokine superfamily, which includes interleukin (IL)-2 and IL-6 (reviewed in Otero et al., 2006). As such, leptin may act directly to affect hematopoiesis and inflammatory responses (Fantuzzi, 2005; Gainsford and Alexander, 1999), and recent evidence suggests that some of the actions of leptin on immune function are mediated by actions on specific pro- and anti-inflammatory cytokines. Genetically engineered mice provide additional evidence for effects of leptin on immunity. Mice with genetically impaired leptin signaling (e.g., *ob/ob* mice, *db/db* mice) show disruptions in immunity (Lord et al., 1998). Specifically, *ob/ob* mice that are unable to produce leptin experience atrophy of lymphatic tissues (e.g., spleen, thymus), and decreases in circulating lymphocyte numbers (Lord et al., 1998). Similarly, *db/db* mice, which are unresponsive to leptin due to a mutation of the leptin

receptor, display immunological deficits. *Db/db* mice have reduced splenic and thymic masses and have a reduced ability to reject skin grafts and to produce cytotoxic responses (Fernandes et al., 1978). Thus, it is clear that leptin has wide effects on immune function, influencing both the maintenance of immune tissues and activation of immune responses.

Leptin has previously been shown to regulate seasonal changes in the humoral immune response (Demas and Sakaria, 2005; Drazen et al., 2001) and energetic trade-offs among the immune and reproductive systems (French et al., 2009a). Yet, it is not clear if leptin may regulate seasonal changes in the APR. There is a growing body of work in the biomedical sciences that suggests that leptin may affect sickness response expression (reviewed in Fantuzzi and Faggioni, 2000; Steiner and Romanovsky, 2007). This work has shown that the connection between leptin and the pro-inflammatory cytokines that are integral to the sickness response is bidirectional. Leptin treatment can enhance the production of pro-inflammatory cytokines by macrophages and in the brain (Loffreda et al., 1998; Luheshi et al., 1999), and LPS and pro-inflammatory cytokine administration triggers increased production of leptin by adipocytes (Faggioni et al., 1998; Finck et al., 1998; Grunfeld et al., 1996; Sarraf et al., 1997).

While it is evident that leptin levels are both modulated by and can modulate pro-inflammatory cytokines, debate remains as to how leptin may influence the actual sickness response. Two separate studies have shown that rats that were administered leptin anti-serum to neutralize circulating leptin levels show a lack of or attenuated fever following LPS injections (Harden et al., 2006; Sachot et al.,

2004); however, Zucker obese rats (*fa/fa*), which have non-functional leptin receptors, show no difference in their fever response to LPS treatment when compared with lean rats, suggesting that leptin signaling is not necessary for fever production (Ivanov and Romanovsky, 2002). Additionally, leptin is a likely candidate mediating the anorexic response to sickness as this hormone mediates food intake in non-sick individuals and is increased in response to infection; however, the evidence linking leptin to the control of sickness-induced anorexia is mixed. Treating rats with leptin antiserum eliminates the anorexic effects of LPS-induced inflammation (Harden et al., 2006; Sachot et al., 2004), suggesting that leptin is critical for anorexic behavior in response to sickness. Alternatively, LPS administration produces the anorexic effect in mice and rats that lack functional leptin signaling due to mutations of the leptin or leptin receptor genes (Faggioni et al., 1997; Lugarini et al., 2005). Therefore, while it remains unclear if and how leptin is modulating sickness responses, if the magnitude of an animal's sickness response is influenced by its available energy stores, then leptin may be neuroendocrine mediator of this response.

Dissertation aims

The overarching goals of this dissertation were to understand how animals respond to changes in energetic state and energetic signals to modulate their investment between reproduction and immunity and how these changes and signals may influence seasonal variation in sickness intensity. In Chapter 1, I focused my efforts toward understanding how variation in different types of metabolic fuels (and their signals) may interact to influence energetic trade-offs between the

reproductive and immune systems. Through this study, I sought to determine how different severities of glucose deprivation may cause female animals to shift energy allocation between the reproductive and immune systems and if increasing a signal fat stores (i.e., leptin) would interact with these trade-offs. In Chapters 2, 3, and 4, I focused my efforts on testing the hypothesis that seasonal variation in sickness response intensity is a product of seasonal changes in energy stores and availability. I modulated energy stores and availability via food restriction and inducing glucose deprivation with 2-DG, respectively, and manipulated energetic signals through treatments with exogenous leptin and insulin. Through these multiple forms of experimental energy manipulation, I was able to start to piece together the role of energy and its signals in the mediation of energetic trade-offs and seasonal modulation of sickness intensity.

Study species

Siberian hamsters have been the focus of much a large portion of the work targeted at understanding the physiological mechanisms that mediate energetic trade-offs and seasonal variation in sickness intensity. Siberian hamsters are seasonally breeding rodents that are highly responsive to changes in photoperiod in the laboratory. When hamsters are housed in long-day (LD), summer-like photoperiods **(**light > 12 hours**),** they remain in reproductive condition and display functioning gonads. However, when housed in short-day (SD), winter-like photoperiods (light < 12 hours**)**, hamsters show regression of their gonads to a non-reproductive state, decreased food intake, and reduced fat stores and circulating leptin levels despite being provided with ad libitum food. With their naturally

occurring changes in energy balance, Siberian hamsters make an excellent study species for understanding the contributions of energy availability and its signals to the regulation of immunity in seasonal contexts.

CHAPTER 1: Metabolic stressors and signals differentially affect energy allocation between reproduction and immune function

Data published in: Carlton, E.D., Cooper, C.L., and Demas, G.E. 2014. Metabolic stressors and signals differentially regulate trade-offs between reproduction and immunity. *General and Comparative Endocrinology* 208: 21-29.

Abstract

Most free-living animals have finite energy stores that they must allocate to different physiological and behavioral processes. In times of energetic stress, trade-offs in energy allocation among these processes may occur. The manifestation of trade-offs may depend on the source (e.g., glucose, lipids) and severity of energy limitation. In this study, we investigated energetic trade-offs between the reproductive and immune systems by experimentally limiting energy availability to female Siberian hamsters (*Phodopus sungorus*) with 2-Deoxy-D-glucose, a compound that disrupts cellular utilization of glucose. We observed how glucoprivation at two levels of severity affected allocation to reproduction and immunity. Additionally, we treated a subset of these hamsters with leptin, an adipose hormone that provides a direct signal of available fat stores, in order to determine how increasing this signal of fat stores influences glucoprivation-induced trade-offs. We observed trade-offs between the reproductive and immune systems and that these trade-offs depended on the severity of energy limitation and exogenous leptin signaling. The majority of the animals experiencing mild glucoprivation entered anestrus, whereas leptin treatment restored estrous cycling in these animals. Surprisingly, virtually all animals experiencing more severe glucoprivation maintained normal estrous cycling throughout the experiment; however, exogenous leptin resulted in lower antibody production in this group.

These data suggest that variation in these trade-offs may be mediated by shifts between glucose and fatty acid utilization. Collectively, the results of the present study highlight the context-dependent nature of these trade-offs, as trade-offs induced by the same metabolic stressor can manifest differently depending on its intensity.

Introduction

Animals are faced with the challenge of obtaining energetic resources. The energy available to an animal under most natural conditions is finite and will depend on the quality and abundance of these energetic resources in the environment. Once an animal obtains energy, it is then faced with the challenge of balancing energy allocation among different physiological, biochemical, and behavioral processes (Ricklefs and Wikelski, 2002; Sheldon and Verhulst, 1996). The balancing of allocation toward diverse biological processes often results in energetic trade-offs among physiological systems. One commonly observed energetic trade-off occurs between the reproductive and immune systems (Demas et al., 2012; Fedorka, 2014), where increased investment into one system results in decreased investment to the other. Maintaining reproduction and activating and maintaining the immune system requires a substantial allocation of energy (Demas, 2004; Speakman, 2008). Therefore, the expression of constraints on these systems and trade-offs between them is often dependent on the animal's access to energetic resources in its environment. This dependence on the environment may explain why in some cases a reproduction-immune trade-off may be observed with

relatively limited resources in the wild but is not observed in the laboratory where food is available ad lib (French and Moore, 2008; French et al., 2009b).

One possibility why the display of energetic trade-offs is often context-dependent is that the display of energetic limitations and trade-offs varies with the type of energy that is being used to fuel the physiological and behavioral processes. Energetic trade-offs may be manifested in different ways depending on an animal's access to both current food availability in the environment and stored energy availability in the form of body fat. For instance, food deprivation inhibits ovulation and estrous behavior in lean, but not fat, female Syrian hamsters (*Mesocricetus auratus*) (Schneider and Wade, 1989), suggesting that having large fat stores may be sufficient to overcome energetic deficits imposed by limited current energetic resources. Food restriction does not suppress antibody production in female Siberian hamsters (*Phodopus sungorus*) (Zysling et al., 2009); however, surgically removing body fat stores (i.e., lipectomy) from male prairie voles and male and female Siberian hamsters results in decreased antibody production (Demas et al., 2003; Demas and Sakaria, 2005). Collectively, these results provide support that different types of metabolic fuels (e.g., glucose from immediate food ingestion, free fatty acids from adipose tissue) may influence the expression of energetic trade-offs within and between these two systems.

One way to manipulate glucose availability is with treatment with 2-Deoxy-D-glucose (2-DG). 2-DG is a non-metabolizable glucose analog, which causes a transient disruption of glycolysis by inhibiting glucose oxidation, resulting in a state of glucoprivation (Horton et al., 1973). Treating ad lib-fed female Syrian hamsters

with high doses of 2-DG (1,750 or 2,000 mg/kg) induces anestrus, whereas a lower dose of 2-DG (750 mg/kg) induces anestrus only in hamsters that are food restricted (Schneider et al., 1993). Previous work in our lab has shown that treatment with 2-DG (750 mg/kg) results in reduced antibody production and reduced reproductive tissue mass in female Siberian hamsters (Zysling and Demas, 2007) and reduced splenocyte production in female deer mice (*Peromyscus maniculatus*) (Demas et al., 1997b). Thus, reducing glucose availability via 2-DG treatment suppresses energetic allocation to both reproduction and immunity.

While it is clear from these studies that reducing glucose utilization can suppress reproductive and immune responses, the context-dependent nature of some of these results (e.g., lower doses of 2-DG suppress reproduction in only food restricted animals, not ad lib-fed animals) illustrates that glucose is not the only fuel source that animals can utilize to power reproduction and immunity. For instance, anestrus can be induced in ad lib-fed hamsters treated with a lower dose of 2-DG (750 mg/kg) if fatty acid utilization is simultaneously blocked (via methyl palmoxirate treatment), suggesting that changes in reproductive status may be driven by availability of all metabolic fuels, rather than just individual metabolic fuel types (Schneider and Wade, 1989). White adipose tissue (WAT), a primary energy source for organisms, provides storage for lipids that can be liberated to free fatty acids and glycerol when triggered by glucagon. WAT is not only a source of fuel, but it is also an endocrine organ that synthesizes and releases hormones (Ahima and Flier, 2000; Cinti, 2007). The peptide hormone leptin is one such adipose hormone, and circulating levels of leptin are directly proportional to the

mass of adipose tissue in mammals (Maffei et al., 1995). Thus, high levels of leptin indicate adequate energy stores, whereas low circulating levels of leptin are consistent with an energy deficit. Furthermore, leptin not only influences lipid metabolism but it also increases glucose metabolism, glucose uptake, glucose turnover, and glucose oxidation (Kamohara et al., 1997).

While leptin was first characterized for its role in food intake and adiposity, there is now ample evidence that leptin plays a role in mediating both reproduction (Caprio et al., 2001; Schneider et al., 2012) and immunity (Carlton et al., 2012; La Cava and Matarese, 2004; Lord, 2002). Treatment with leptin restores estrous cycling in fasted female Syrian hamsters, however, when hamsters are fasted and treated with 2-DG, leptin does not restore estrous cycling. As 2-DG inhibits glucose oxidation, these results suggest that leptin influences energy allocation to reproduction via effects on metabolic fuel oxidation rather than through signaling of available adipose stores (Schneider et al., 1998). Additionally, leptin treatment counteracts the fasting-induced suppression of cell-mediated immunity in mice (Lord et al., 1998), and it attenuates the suppressive effects of surgical lipectomy on antibody production in male Siberian hamsters (Demas and Sakaria, 2005). Male Siberian hamsters treated with 2-DG show reductions in antibody production, and providing exogenous leptin alleviates this suppression of humoral immunity (Drazen, 2001) suggesting that the effects of leptin on humoral immunity are at least in part mediated through changes in signals of fat availability (Drazen, 2001; Drazen et al., 2001). Therefore, leptin may act differently in how it regulates energy allocation to reproduction versus immunity when glucose utilization is impaired.

The goal of the present study was to determine how leptin, as a neuroendocrine signal, affects energy allocation between the reproductive and immune systems in female Siberian hamsters experiencing glucoprivation. Specifically, we experimentally reduced glucose utilization with either a low (750 mg/kg) or high dose (1750 mg/kg) of 2-DG, and then supplemented animals with exogenous leptin. We assessed reproductive (i.e., estrous cycling, reproductive tissue mass) and immune (i.e., serum bacterial killing, antibody production) indices in response to our treatments. We predicted that mild glucoprivation (low 2-DG dose) would reduce reproductive tissue mass and antibody production and that leptin treatment should restore antibody production and may restore reproductive tissue mass. Additionally, we predicted that at more severe glucoprivation (high 2-DG dose), animals would be more energy limited and would show halted estrous cycling in addition to reduced reproductive tissue mass, antibody production, and bacterial killing ability. Because the high dose of 2-DG provides significant energy limitation, we expected that leptin supplementation would only provide a sufficiently large energetic signal to restore one system (e.g., the immune system because it is most important for survival) and this restoration might occur at a potential additional energetic cost to the reproductive system. Finally, in order to assess potential causation behind trade-offs, we assessed serum triglyceride and cortisol concentrations to determine the physiological mechanisms that may mediate energy allocation.

Methods

Animals and housing

Adult female (> 60 days of age) Siberian hamsters (n=58) were obtained from our breeding colony at Indiana University. The progenitors of these animals were generously provided by Dr. Randy Nelson (Ohio State University) and Dr. Timothy Bartness (Georgia State University). In order to minimize the effects of inbreeding, our animals are outbred approximately every 10 generations. All animals were initially group housed (2-5 per cage with same sex siblings on weaning at 17-18 days of age) in long-day photoperiods (light:dark, 16:8). Temperature (20 ± 2°C) and humidity (50 ± 10%) were maintained at constant levels. For the experiment, animals were all housed in the same room where they were maintained on long days (16:8) and individually housed in polypropylene cages (27.8 x 17.5 x 13.0 cm). Food (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO, USA) and tap water were available ad lib during the entire course of the experiment. Animals used in this experiment came from 18 different litters across 8 different breeding pairs. All animal methods were reviewed and approved by the Institutional Animal Care and Use Committee at Indiana University Bloomington (protocol no. 10-038).

Experimental methods

For eight days prior to experimental treatments, vaginal cell samples were obtained by vaginal lavage (with 0.9% sterile saline) from each animal between 0930h and 1130h EST to determine estrous cycle stage (Scotti et al., 2007). For five

days prior to experimental treatments, body mass (to the nearest 0.1g) and food consumption (to the nearest 0.1g) were assessed daily between 0930h and 1130h EST to establish pre-treatment average body mass and average food intake baseline values. Food consumption was assessed by weighing the food pellets remaining in the hopper each day. Daily vaginal sampling and body mass and food consumption measurements were continued throughout the entirety of the experiment.

Animals were initially quasi-randomly selected (i.e., no more than one animal from each litter was placed into a single group) and assigned to one of three 2-DG treatment groups that received 0.1 ml injections of either a 0.9% sterile saline control or of one of two concentrations of 2-DG (750 mg/kg dissolved in 0.9% sterile saline or 1750 mg/kg dissolved in 0.9% sterile saline; Sigma-Aldrich, St. Louis, MO, USA), administered intraperitoneally (i.p.). The 750 mg/kg dose was used because a previous study in Siberian hamsters showed that this dose results in decreased antibody production and reduced reproductive tissue mass (Zysling and Demas, 2007). The 1750 mg/kg dose was used because this dose is the lowest dose of 2-DG that induces anestrus in ad lib-fed Syrian hamsters (Schneider et al., 1993). Additionally, these concentrations are lower than doses that induce torpor in Siberian hamsters (2,500 mg/kg) (Dark et al., 1994; Zysling and Demas, 2007). Within each 2-DG treatment group, animals were further divided into one of two groups that received either control injections of 0.9% sterile saline or leptin. Control animals received a 0.2 ml injection of 0.9% sterile saline, while leptin-treated animals received a 0.2 ml injection of 45 μg recombinant murine leptin (Peprotech Inc., Rocky Hill, NJ, USA) dissolved in 0.9% sterile saline, administered i.p.

Injections of 2-DG (or saline control) were administered every other day for 12 days, for a total of 6 injections while injections of leptin (or saline control) were administered every day for 12 days, for a total of 12 injections. All injections were provided between 1400 and 1500 h EST. Final group sample sizes were as follows: Veh/Veh (n=10), Veh/Lep (n=10), Low-2DG/Veh (n=10), Low-2DG/Lep (n=8), High-2DG/Veh (n=10), High-2DG/Lep (n=10).

On the day following the first 2-DG or saline injection, all animals received a single subcutaneous injection of 100 µg of keyhole limpet hemocyanin (KLH; Sigma-Aldrich, St. Louis, MO, USA) suspended in 0.1 ml sterile saline in order to generate a humoral immune response. KLH is an innocuous respiratory protein derived from the giant keyhole limpet (*Megathura crenulata*) that generates a robust, non-replicating antigenic response in rodents, but does not make the animals sick (i.e., no inflammation or fever) (Dixon et al., 1966).

Blood sampling and necropsies

On days 5 and 10 post KLH injection, a blood sample was drawn from all animals via the retro-orbital sinus for immediate measurement of blood glucose and later measurement of bacterial killing ability (days 5 and 10), KLH-specific antibodies (days 5 and 10), serum triglyceride concentrations (day 5), and serum cortisol concentrations (day 10). These time points were chosen to capture peak anti-KLH immunoglobulin G production during the course of the immune response (Demas et al., 1997a). Triglyceride concentrations and cortisol concentrations were only measured at single time points during the study due to limited serum

availability. We chose to measure triglycerides at day 5 because animals that stopped estrous cycling did so no later than this date, so any effects of triglycerides on this measure could ideally be captured at this date. We assessed cortisol at day 10 because a previous study in our lab showed that cortisol concentrations do not differ in Siberian hamsters treated with 750 mg/kg 2-DG from day 5 to day 10 (Zysling and Demas, 2007). Briefly, animals were lightly anesthetized with isoflurane vapors and blood samples were drawn from the retro-orbital sinus between 1300 and 1400 h EST. Blood samples were allowed to clot at room temperature for 1 h, the clots were removed, and the samples were centrifuged at 4°C for 30 min at 2500rpm. Serum aliquots were aspirated and stored in sealable polypropylene microcentrifuge tubes at -20°C until assayed. Each blood sample consisted of ~3.5% of the animal's total blood volume, and all blood samples were collected within 3 minutes of initial handling. Animals were euthanized and necropsies were performed at the completion of the study (day 11 post KLH injection). Uterine horns, ovaries, inguinal WAT (IWAT), parametrial white adipose tissue (PWAT), and retroperitoneal WAT (RWAT) were removed to determine the effects of 2-DG and leptin on these tissues. All tissues were cleaned of connective tissues and weighed to the nearest 0.1 mg. A composite adipose tissue score was calculated by summing the individual WAT pad masses.

Blood glucose measurement

Blood glucose levels were measured from the blood samples collected at 5 and 10 days after KLH injection. Immediately upon sampling, ~5 µl of whole blood

was transferred onto the test strips of a blood glucose monitoring system (ReliOn, Micro Blood Glucose Monitoring System, Arkray USA, Inc., Minneapolis, MN, USA), and the readout was recorded. The meter was previously calibrated using an internal standard provided by the manufacturer.

Vaginal cytology

After vaginal cell samples were obtained via vaginal lavage, samples were transferred to microscope slides, fixed with methanol, and stained with Giemsa. These samples were evaluated for estrous stage (diestrus I, diestrus II, proestrus, estrus) under 100x magnification. The following cellular characteristics were used to characterize the four stages of the estrous cycles: diestrus I (presence of many polymorphonuclear leucocytes and some nonnucleated keratinized cells), diestrus II (primarily secretory material and cellular debris, some parabasal cells), proestrus (clumps of lightly staining nucleated epithelial cells), estrus (many nonnucleated keratinized cells) (Moffatt-Blue et al., 2006; Scotti et al., 2007). In addition, anestrus was characterized by slides which contained a lot of cellular debris with cells that did not appear intact. Animals were determined to have stopped cycling if, after they had previously been cycling in the experiment, they entered anestrus and remained in anestrus until completion of the experiment. Two animals (one in the low dose 2-DG and vehicle group; one in the high dose 2-DG and leptin group) did not show normal estrous cycling during the eight days prior to the start of injections, so they were excluded from the cycling analysis.

Bacterial killing

We used an ex vivo bacterial killing assay as a functional assessment of the innate immune system's ability to clear a relevant pathogen. This assay quantifies the relative number of *Escherichia coli* colony forming units (CFU) that grow after incubation with serum. Briefly, lyophilized *E. coli* (Epower^{TM}, ATCC #8739, Microbiologics, St. Cloud, MN, USA; 1 pellet = 10^7 CFU) was added to 40 ml 1 M sterile PBS warmed to 37°C to create a bacterial stock solution. This solution was activated by incubation for 30 min at 37°C. The stock bacteria solution (500,000 CFU/ml) was diluted 1:10 with sterile 1 M PBS to create a 50,000 CFU/ml working solution. Meanwhile, serum samples were diluted 1:20 in glutamine enriched CO_2-independent media (Invitrogen Corp., Carlsbad, CA, USA). For each sample, the bacterial working solution was added at a 1:10 ratio to the diluted serum sample. To generate a positive control (i.e., solution containing only media and bacteria), the bacterial working solution was diluted 1:10 with glutamine enriched CO_2-independent media. The diluted samples and the positive control were incubated for 30 min at 37°C to induce bacterial killing. After incubation, 50 µl of sample and the positive control was added to tryptic soy agar plates in duplicate. All plates were covered, inverted, and stored overnight at 37°C. Following incubation, colony numbers were counted on each plate, and duplicates were averaged. Bactericidal capacity was calculated as a percent of bacteria killed relative to the positive control plates in which no killing occurred.

Anti-KLH enzyme-linked immunosorbent assay (ELISA)

To assess humoral immunity to KLH injection, serum anti-KLH immunoglobulin G (IgG) concentrations were assayed using an enzyme-linked immunosorbent assay (ELISA) (Demas et al., 2003). Microtiter plates were coated with KLH by incubating overnight at 4°C with 0.5 mg/ml KLH in sodium bicarbonate buffer (pH 9.6). Plates were washed with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T; pH 7.4), then blocked with 5% non-fat dry milk in PBS (to reduce non-specific binding), and then washed again with PBS-T. Thawed serum samples were diluted 1:20 with PBS-T, and 300 µL of each serum dilution was added to the plate wells in duplicate. Positive control samples (i.e., pooled sera from hamsters previously shown to have high anti-KLH antibody responses) and negative control samples (i.e., pooled sera from KLH-naïve hamsters) were also diluted 1:20 with PBS-T and added to the plate wells in duplicate. Plates were incubated at 37°C for 3 h and then washed with PBS-T. 150 µL of secondary antibody (alkaline phosphatase-conjugated-anti Syrian hamster IgG diluted 1:500 with PBS-T; Rockland, Gilbertsville, PA, USA) was added to the wells and the plates were incubated for 1 h at 37°C. Plates were then washed again with PBS-T and 150 µL of the enzyme substrate *p*-nitro-phenyl phosphate (Sigma-Aldrich, St. Louis, MO, USA; 0.1 mg/ml in diethanolamine substrate buffer) was added to each well. The absorbance of each well was measured (Bio-Rad iMark Microplate Reader, Hercules, CA, USA) at 405 nm. The mean for each sample was calculated and expressed as a percentage of the positive control mean (% plate positive).

Triglyceride assay

Total serum triglyceride concentrations were assessed with a commercially prepared colorimetric assay kit (Triglyceride Colorimetric Assay Kit, Cayman Chemical Co., Ann Arbor, MI, USA). Prior to testing samples, we validated the appropriate dilution (1:4) so that triglyceride concentrations lay within the detectable range of the assay. Some samples expressed triglyceride levels at the 1:4 dilution that ran off the curve, so these samples were rerun at a 1:10 dilution. Intra-assay variabilities were 3.2% and 3.9%; inter-assay variability was 0.7%.

Cortisol enzyme immunoassay (EIA)

Cortisol is the predominant glucocorticoid in Siberian hamsters, with concentrations ~100x that of corticosterone (Reburn and Wynne-Edwards, 2000). Serum cortisol concentrations were determined in multiple enzyme immunoassays (EIAs) from a commercially prepared kit (Cortisol EIA Kit; Enzo Life Sciences, Inc., Farmingdale, NY, USA). This assay was previously validated for use in Siberian hamsters (Demas et al., 2004) and is highly specific for cortisol; cross-reactivity with corticosterone is 27.7% and <4.0% for other steroid hormones. The sensitivity of the assay is 56.72 pg/ml. Samples were diluted to 1:40 with assay buffer and run in duplicate. Intra-assay variabilities were 3.0% and 3.5%; inter-assay variability was 14.0%.

Statistical analyses

All statistical tests were performed using JMP 10 (SAS Institute Inc., Cary, NC, USA), and a value of $P < 0.05$ was considered to be statistically significant. Data were checked for normality and homogeneity of variance and those data that were non-normally distributed were transformed. Glucose, cortisol, and triglyceride concentrations were not normally distributed and were log transformed to best meet the assumptions of parametric tests. Bacterial killing ability was not normally distributed and was reverse square root transformed to meet the assumptions of parametric tests. All differences in measurements collected at only one time point (i.e., fat tissue masses, reproductive tissue masses, triglyceride concentrations, cortisol concentrations) were assessed via a two-way (2-DG treatment (3) x leptin treatment (2)) analysis of variance (ANOVA). Body mass at the time of sampling was included as a covariate in the models for the fat tissue masses, reproductive tissue masses, and triglyceride concentrations to control for the effect of body mass on these dependent variables. Differences in repeated measures (i.e., body mass, food intake, antibody levels, bacterial killing ability, glucose concentrations) were assessed via repeated-measures ANOVAs with time as a within-subjects variable. Initial body mass was included as a covariate in the model for food intake levels to control for the effect of body mass on this measure. The within-subject comparisons for body mass and food intake violated assumptions of sphericity and were Greenhouse-Geisser (GG)-corrected. Treatment effects on the frequencies of animals remaining in estrus throughout the experiment were assessed with Fisher's Exact Test because expected values in

some cells were less than 5. Post-hoc comparisons between pair-wise means were conducted using Fisher's LSD tests when the overall ANOVAs were statistically significant.

Results

Body mass, food intake, and body fat

There were no effects of 2-DG ($F_{2,52}$ = 0.63, P = 0.535; Table 1) or leptin ($F_{1,52}$ = 0.62, P = 0.434) on final body mass. While treatment did not affect final body mass, body mass decreased over the course of the experiment (within subjects, $F_{1.9,103.9}$ = 8.89, P < 0.001, GG-corrected). Food intake over the course of the experiment was related to the initial body mass of the animals ($F_{1,51}$ = 13.89, P < 0.001) After controlling for the effect of body mass, there were no effects of 2-DG ($F_{2,52}$ = 1.02, P = 0.369; Table 1) or leptin ($F_{1,52}$ = 0.06, P = 0.812) on daily food intake. Additionally, food intake did not change over the course of the experiment (within subjects, $F_{7.7,393.1}$ = 1.27, P = 0.258, G-G-corrected). IWAT, PWAT, RWAT, and composite adipose tissue mass were all related to the body mass of the animal at the time of tissue collection (P < 0.001 in all cases). After controlling for the effect of body mass, there were no treatment effects on any of these fat tissue measures (P > 0.05 in all cases; Table 1).

Blood glucose

Blood glucose levels did not differ across sampling times within individuals ($F_{1,51}$ = 2.38, P = 0.129; Table 1), nor did levels differ among groups (2-DG, $F_{2,51}$ = 0.12, P = 0.886; leptin, $F_{1,51}$ = 0.16, P = 0.693; 2-DG x leptin, $F_{2,51}$ = 0.58, P = 0.564).

Table 1. Effects of 2-DG dose and leptin treatment on mean (±SEM) final body mass, food intake on the last day of the experiment, composite body fat mass, blood glucose levels, and paired ovary mass. No statistically significant differences between group means (P <0.05) were found for any of these measures.

	Vehicle	Leptin
Final body mass (g)		
Vehicle	38.7 ± 1.9	36.1 ± 2.3
Low	40.1 ± 2.4	37.9 ± 2.4
High	38.9 ± 1.6	41.0 ± 3.0
Food Intake (g/day)		
Vehicle	4.8 ± 0.2	4.7 ± 0.5
Low	5.0 ± 0.5	4.9 ± 0.4
High	4.7 ± 0.2	4.7 ± 0.3
Composite Body Fat (g)		
Vehicle	1.59 ± 0.21	1.43 ± 0.31
Low	1.97 ± 0.25	1.55 ± 0.30
High	1.55 ± 0.19	1.83 ± 0.35
Day 10 Blood Glucose (mg/dl)		
Vehicle	115.3 ± 6.5	113.2 ± 9.2
Low	110.4 ± 4.3	120.5 ± 7.2
High	113.3 ± 6.3	106.9 ± 5.4
Paired Ovary Mass (mg)		
Vehicle	18.40 ± 2.69	18.36 ± 0.58
Low	21.95 ± 2.69	14.80 ± 1.22
High	18.37 ± 1.61	23.23 ± 2.97

Estrous cycling

Estrous cycling was impaired in animals treated with the low 2-DG dose (Figure 1A). Fifty-six percent of the animals that received the low 2-DG dose became anestrus by the end of the experiment, whereas in all other groups, 20% or fewer of the animals were anestrus at the end of the experiment. This difference in anestrus between the low 2-DG dose group and the other five groups was significant ($P = 0.003$).

Reproductive tissue masses

Uterine horn and ovary mass were affected by the body mass of the animal at the time of tissue collection (uterine horns: $F_{1,51} = 7.36$, $P = 0.009$; ovaries: $F_{1,51} = 8.68$, $P = 0.005$). After controlling for body mass, there were effects of both 2-DG ($F_{2,51} = 3.36$, $P = 0.024$) and leptin ($F_{1,51} = 6.60$, $P = 0.013$) but no effect of the 2-DG x leptin interaction ($F_{2,51} = 2.25$, $P = 0.116$) on uterine horn mass (Figure 1B). Specifically, uterine horn masses in all treatment groups were significantly lower than those of the vehicle-treated controls (for all comparisons, $T \geq 2.36$, $P \leq 0.022$). Conversely, there were no treatment effects on ovary mass (2-DG, $F_{2,51} = 0.59$, $P = 0.558$; leptin, $F_{1,51} = 0.06$, $P = 0.811$; 2-DG x leptin, $F_{2,51} = 2.92$, $P = 0.063$; Table 1).

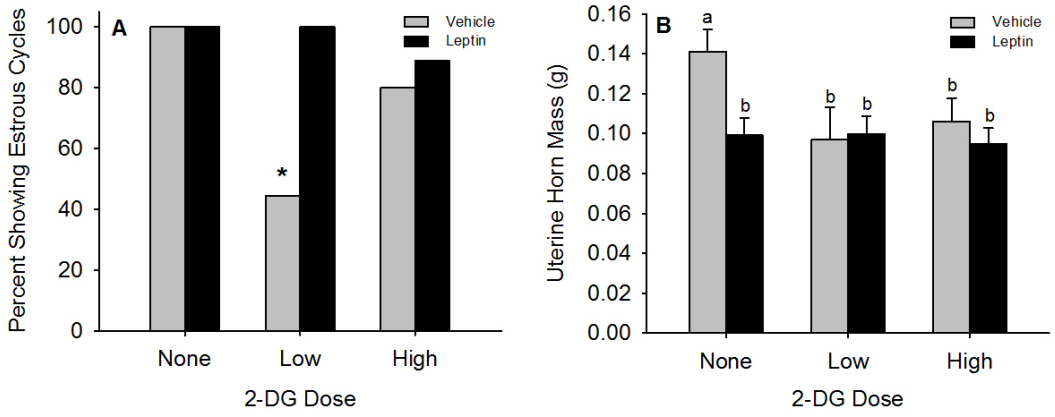

Figure 1. Effects of 2-DG dose and leptin treatment on (**A**) the percentage of hamsters showing normal estrous cycles and (**B**) mean (±SEM) uterine horn mass. In figure **A**, groups that differ significantly ($P < 0.05$) from the vehicle/vehicle-treated control are indicated by an asterix (*). In figure **B**, groups with different letters indicate statistically significant differences between group means ($P < 0.05$); groups sharing the same letter are statistically equivalent.

Antibody production

In all groups, anti-KLH IgG levels increased from Day 5 to Day 10 (within subjects, $F_{1,52} = 64.34$, $P < 0.001$; Figure 2A). There was a main effect of 2-DG dose (between subjects, $F_{2,52} = 3.37$, $P = 0.042$), such that animals treated with the high dose of 2-DG had lower IgG levels than animals treated with just the vehicle ($P = 0.012$). There were no main effects of leptin ($F_{1,52} = 2.01$, $P = 0.162$) or the 2-DG x leptin interaction ($F_{2,52} = 2.79$, $P = 0.071$) on IgG levels. Specifically, this high 2-DG suppression of IgG levels was driven by animals treated with the high 2-DG dose and leptin showing lower IgG levels than animals treated only with leptin (and no 2-DG) (day 5, $T = 2.99$, $P = 0.004$; day 10, $T = 2.79$, $P = 0.007$).

Bacterial killing

Bacterial killing ability did not change across blood sampling time points (within subjects, $F_{1,52}$ = 0.16, P = 0.688; Figure 2B). However, there was a main effect of leptin ($F_{1,52}$ = 11.37, P = 0.001), but no effects of 2-DG ($F_{2,52}$ = 1.36, P = 0.266) or the 2-DG x leptin interaction ($F_{2,52}$ = 0.16, P = 0.0755) on bacterial killing ability. Specifically, at both sampling points, bacterial killing was elevated in animals treated with leptin but no 2-DG as compared to the vehicle-treated controls (day 5, T = 2.54, P = 0.014; day 10, T = 2.29, P = 0.026).

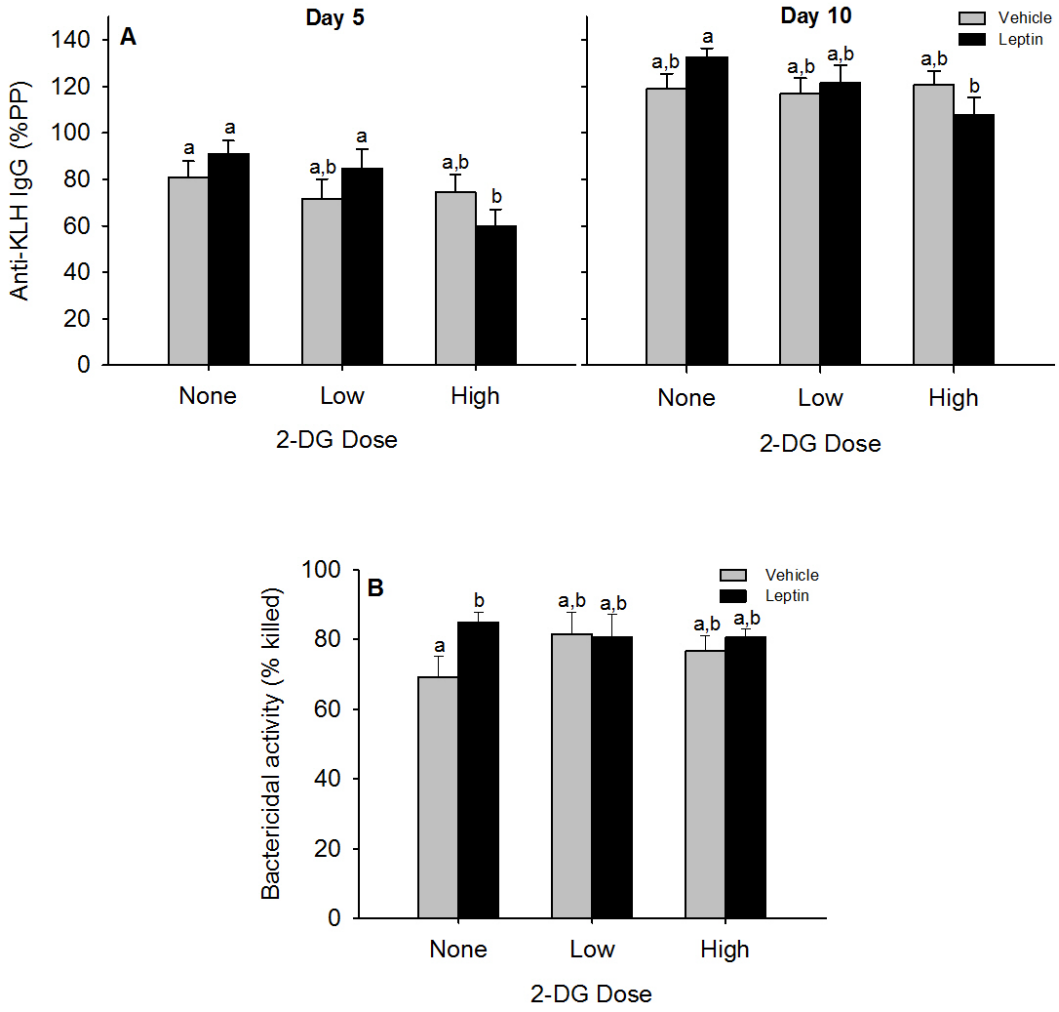

Figure 2. Effects of 2-DG dose and leptin treatment on mean (±SEM) (**A**) serum anti-KLH immunoglobulin G (IgG) at day 5 (left) and day 10 (right) after KLH inoculation and (**B**) serum bacterial killing ability at day 10 after KLH inoculation (as there was no effect of time on this measure, only data from day 10 are shown). Groups with different letters indicate statistically significant differences between group means (P <0.05); groups sharing the same letter are statistically equivalent.

Serum triglycerides

Serum triglyceride levels were positively related to the body mass of the animal at the time of sampling ($F_{1,57}$ = 11.53, P = 0.001). After controlling for the effect of body mass, there was an effect of 2-DG ($F_{2,57}$ = 5.18, P = 0.009; Figure

3A), but no effects of leptin ($F_{1,57}$=3.51, P=0.067) or the 2-DG x leptin interaction ($F_{2,57}$ = 0.88, P = 0.421) on triglyceride levels. Specifically, animals treated with the high 2-DG dose and either vehicle (T = 3.07, P = 0.004) or leptin (T = 3.36, P = 0.002) showed decreased triglyceride levels as compared to vehicle-treated controls.

Serum cortisol

Cortisol did not differ among groups (Figure 3B). There were no significant effects of 2-DG ($F_{2,50}$ = 1.67, P = 0.199), leptin ($F_{1,50}$ = 0.06, P = 0.805), or the 2-DG x leptin interaction ($F_{2,50}$ = 0.16, P = 0.854) on serum cortisol levels.

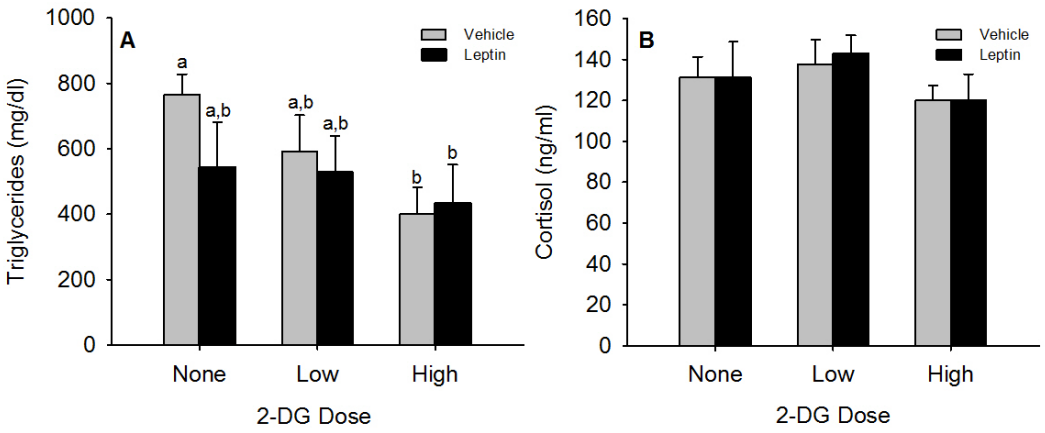

Figure 3. Effects of 2-DG dose and leptin treatment on mean (±SEM) (**A**) serum triglyceride concentrations at day 5 after KLH inoculation and (**B**) serum cortisol concentrations at day 10 after KLH inoculation. Groups with different letters indicate statistically significant differences between group means (P <0.05); groups sharing the same letter are statistically equivalent.

Discussion

Our findings demonstrate key trade-offs between the reproductive and immune systems in female Siberian hamsters and that these trade-offs depend on the severity of energy limitation and exogenous leptin signaling. We found that the majority of the animals experiencing mild glucoprivation (low dose 2-DG) entered anestrus, whereas leptin treatment restored estrous cycling in these animals. Surprisingly, virtually all animals experiencing more severe glucoprivation (high dose 2-DG) maintained normal estrous cycling throughout the experiment and did not show anestrus. In addition, we found that animals experiencing any level of glucoprivation displayed reduced uterine horn mass and exogenous leptin not only did not restore this mass, but it may have contributed to its decrease. In contrast to previous work in this species (Zysling and Demas, 2007), mild glucoprivation did not suppress antibody production in the current study. Alternatively, we found that animals experiencing more severe glucoprivation while receiving leptin supplementation showed reduced antibody production at both days 5 and 10 after KLH inoculation. The measure of innate immunity, serum bacterial killing ability, was not affected by our glucoprivation treatment, although leptin treatment may have enhanced this measure. Taken together, our results suggest that at mild glucoprivation, female Siberian hamsters show reduced energy allocation to the reproductive system (via inhibition of estrous cycling) but that this reduction in energy allocation can be alleviated with an increased signal of fat stores. At a more severe level of glucoprivation, female Siberian hamsters do not reduce energy allocation to estrous cycling; however, providing these animals with a simultaneous

signal of increased fat stores results in decreased antibody production, suggesting that these competing signals of energy availability may be resulting in energy being allocated to other physiological processes or systems not assessed in this study (e.g., thermoregulation, motor activity, self-maintenance).

In order to understand the potential mechanisms mediating energy allocation to the reproductive and immune systems during glucoprivation and leptin supplementation, we assessed circulating cortisol and triglyceride concentrations during the experiment. We measured circulating cortisol levels because 2-DG is often used in experimental procedures to induce metabolic stress, as its glucoprivic effects are often accompanied by increases in circulating glucocorticoid levels (Demas et al., 1997b; Weidenfeld et al., 1994). Stress can have both enhancing (e.g., acute stress) and suppressive (e.g., chronic stress) effects on immunity, while it acts mainly to suppress reproduction (Dhabhar, 2002; Sapolsky et al., 2000; Weil and Nelson, 2012). We found no differences in cortisol levels among the groups in our study. Similarly, previous studies in female Siberian hamsters found no differences in cortisol levels between animals treated with a 750 mg/kg dose of 2-DG as compared to controls (Zysling and Demas, 2007) and between hamsters food restricted to 70% of ad lib intake in comparison to controls (Zysling et al., 2009). Chronic stress from our daily injections could have contributed to the lack of differences in cortisol (and blood glucose) among groups, as all animals may have shown elevated baseline cortisol levels as a product of injection-related stress. The cortisol values measured in this study are ~50 ng/ml higher than those of female Siberian hamsters who have not been given daily injections or daily handling

(Zysling et al., 2009). However, this large difference in cortisol levels between the hamsters in our study and the hamsters in the earlier study (Zysling et al., 2009) is more likely due to changes in the sensitivity of the cortisol antibody, as a previous study applying similar injection and handling methods to ours found cortisol levels similar to baseline levels of non-injected animals (Zysling and Demas, 2007). Additionally, it is possible that we did not see differences in cortisol levels among the groups because we took blood samples prior to the day's injection of 2-DG (i.e., 23 hours from the last injection of 2-DG), and cortisol levels were not chronically elevated by our 2-DG treatment. Alternatively, the differential effects of our treatments on reproduction and immunity may not be caused by glucocorticoids but rather may be mediated by the utilization of different energy forms or energy reallocation.

We measured serum triglyceride levels to assess whether animals were potentially tapping into lipid energy stores while their glucose stores were less accessible during glucoprivation. We were motivated to probe this mechanism when we discovered that estrous cycling was only suppressed in the group receiving the low 2-DG dose and not in the group that received the high 2-DG dose. This result is in contrast to work in Syrian hamsters that showed that in animals that are provided ad lib access to food, estrus is only suppressed when animals receive a dose of at least 1,750 mg/kg 2-DG and not suppressed in animals receiving a dose of 750 mg/kg (Schneider et al., 1993). One hypothesis for why we observed no suppression of estrous cycling in hamsters treated with the high 2-DG dose could be that this high dose inhibited glucoprivation so greatly that it required animals to

shift their main source of fuel to free fatty acids (FFA). It is well established that when oxidation of one fuel type is inhibited, oxidation of the other fuel type is enhanced (Friedman, 1998), and FFAs can be used to fuel reproduction and immunity (Pond, 1996; Schneider, 2004). Furthermore, administration of 2-DG results in increases in circulating FFA in rats and Siberian hamsters (Brito et al., 2008; Yamamoto et al., 1984). Consistent with this hypothesis, animals that were treated with the high 2-DG dose had the lowest levels of circulating triglycerides, possibly indicating that these animals were showing greater breakdown of triglycerides into FFA and glycerol (i.e., undergoing lipolysis). If hamsters experiencing more severe glucoprivation increase lipolysis to a greater extent than hamsters experiencing less severe glucoprivation, this increase in FFA availability may be great enough in the high 2-DG group to fuel estrous cycling despite the reduction in useable glucose. Furthermore, in ad lib fed Syrian hamsters, estrous cycling is only halted in animals experiencing both glucoprivation and lipoprivation, not in animals that are only experiencing one of the two (Schneider and Wade, 1989). Thus, it is possible that an increase in lipolysis could have counteracted the estrus-suppressing effects of glucoprivation.

While estrous cycling was suppressed in hamsters treated with the low 2-DG dose, concurrent treatment with leptin restored cycling. This result is in contrast to work in Syrian hamsters that shows that leptin does not restore normal estrous cycling in animals that are fasted while receiving treatment with 2-DG (Schneider et al., 1998). However, our study differs from this study because our animals were fed ad lib, and leptin treatment restores normal estrous cycling in Syrian hamsters

that are fasted but do not receive 2-DG (Schneider et al., 1998). Since leptin supplementation cannot override the suppressive effects of glucoprivation on estrous cycling in fasted animals, Schneider and colleagues suggest that leptin influences reproductive function by indirectly affecting fuel oxidation (i.e., the "metabolic hypothesis"), not by acting as a signal of available fat stores (Schneider et al., 1998; Schneider et al., 2012; Schneider and Zhou, 1999). In non-food restricted animals, however, it seems plausible that leptin supplementation may be able to overcome some of the energy limitations of glucoprivation since leptin increases intracellular oxidation of fatty acids (Shimabukuro et al., 1997). Thus, in our study, because animals were not fasted while being treated with 2-DG, leptin treatment may have been able to increase intracellular oxidation of fatty acids enough so as to compensate for the estrus-suppressing effects of reduced intracellular glucose oxidation at the low dose of 2-DG (although if entirely the case, we may have seen lower triglyceride levels in the group that received low 2-DG and leptin).

It is not surprising that our measure of innate immunity, serum bacteria killing, was not affected by any of our treatments, as maintaining the innate immune system comes at a fairly low energetic cost (Klasing, 2004). We did expect that 2-DG treatment (at both doses) would result in decreased antibody production due to the energetic costs of antibody generation, but instead, we found that 2-DG treated animals only showed a reduction in anti-KLH IgG production at the high dose. It is possible that, although we did not see reduced IgG production at the low dose, we may have seen reduced production of a different antibody type that we did not

measure (e.g., IgM). It is also possible that because energy was allocated away from investment in reproduction (i.e., all groups experiencing glucoprivation showed decreased uterine horn mass), then energy allocation toward humoral immunity could be maintained. For instance, other work in Siberian hamsters has shown that males that are treated with 2-DG show decreased testes mass but no reduction in delayed-type hypersensitivity (DTH) immune response, while females show no reduction in uterine mass but do show decreased DTH response (Martin et al., 2008a). In our study, the group that received the high dose of 2-DG and leptin showed a reduction in IgG levels as compared to the group that only received leptin. This result was contrary to our predictions but may be the result of leptin treatment causing energy to be allocated to other energetically-costly physiological processes that we did not quantify in this study (e.g., thermoregulation, motor activity, self-maintenance). For example, treating pregnant Siberian hamsters with leptin results in increased allocation to reproduction but a subsequent decrease in innate immune function (French et al., 2009a). In our study, it is quite possible that leptin treatment increased energy allocation to another physiological process. This shunting of energy toward another process, coupled with already limited energy due to glucoprivation, may have resulted in less energy available for immune function. These results highlight the importance of expanding our investigation from dichotomous trade-offs to trade-offs among several systems in future studies of this sort.

It is important to note that much of the work on energetic regulation of estrous cycling has been performed in Syrian hamsters. Syrian and Siberian

hamsters are both seasonally breeding rodents that reproduce in long-day lengths and shut down reproduction in short photoperiods; however, Syrian hamsters lose weight while transferring from short to long photoperiods, while Siberian hamsters gain weight during this transition (Bartness and Wade, 1985). In addition, these species show differences in how 2-DG treatment affects other metabolically-sensitive processes like food consumption and torpor (Bartness et al., 1995; Dark et al., 1994; Ritter and Balch, 1978; Schneider et al., 1993). Thus, it is not surprising that these animals may show differences in their reproductive responses to glucoprivation. In our study and a previous study from our lab (Zysling and Demas, 2007), we induced glucoprivation by injecting animals with 2-DG every other day. Alternatively, many of the studies that we have cited in this paper have induced glucoprivation by injecting animals with 2-DG more frequently (e.g., every six hours over the course of two days (Schneider et al., 1993); every 24 hours for three consecutive days (Demas et al., 1997b)). These differences in the frequency of injections may be reflected in the differences in the effects of glucoprivation across these studies. Although we provided 2-DG less frequently than in some of these other studies, we are still confident that we were inducing glucoprivic effects because this same injection protocol has been previously shown to cause glucoprivation and affect immune responses in rats at a lower dose than the doses we chose (Chou et al., 1996).

In conclusion, our data support the presence of trade-offs between the reproductive and immune systems, which are regulated distinctly based upon the severity of glucoprivation and leptin signaling that the animal experiences. Because

varying components of the reproductive and immune systems were suppressed under different levels of glucoprivation, these results highlight how trade-offs induced by the same energetic stressor can manifest in opposite ways depending on the intensity of the stressor. In addition, the context-dependent nature of these trade-offs support the idea that animals should preferentially allocate energy to processes that support survival or reproduction based upon their internal energetic environment. Collectively, these data showcase the complexities underlying the metabolic mechanisms facilitating energetic trade-offs and highlight that future studies should examine trade-offs among several physiological systems to elucidate how metabolic fuels are being allocated during energetic stress.

CHAPTER 2: Leptin mediates seasonal variation in some but not all symptoms of sickness in Siberian hamsters

Data published in: Carlton, E.D. and Demas, G.E. 2014. Leptin mediates seasonal variation in some but not all symptoms of sickness in Siberian hamsters. *Hormones and Behavior* 66: 802-811.

Abstract

Many seasonally breeding species, including Siberian hamsters (*Phodopus sungorus*), exhibit seasonal variation in sickness responses. One hypothesis regarding the mechanism of this variation is that sickness intensity tracks an animal's energetic state, such that sickness is attenuated in the season that an animal has the lowest fat stores. Energetic state may be signaled via leptin, an adipose hormone that provides a signal of fat stores. Siberian hamsters respond to extended housing in short, winter-like days by reducing fat stores and leptin levels, relative to those housed in long, summer-like days. Sickness responses are also attenuated in short-day hamsters as compared to long-day hamsters. We hypothesized that leptin provides a physiological signal by which seasonally breeding animals modulate sickness responses, such that animals with higher leptin levels show increased sickness intensity. To test this, we provided short-day hamsters with a long-day-like leptin signal and assessed their responses to lipopolysaccharide (LPS), a sickness-inducing antigen. We compared these responses to short-day vehicle-, long-day vehicle-, and long-day leptin-treated hamsters. Unexpectedly, LPS induced a hypothermic response (rather than fever) in all groups. Short-day vehicle-treated hamsters exhibited the greatest LPS-induced hypothermia, and leptin treatment attenuated this response, making hypothermia more long-day-like. Contrary to our hypothesis, short-day leptin-treated hamsters showed the least

pronounced LPS-induced anorexia among all groups. These results suggest that leptin may mediate some but not all aspects of seasonal sickness variation in this species. Future studies should be targeted at determining roles of other energetic hormones in regulating seasonal sickness response variation.

Introduction

Seasonally breeding animals must respond to temporal changes in environmental factors like climate fluctuations, social interactions, and resource availability. While their abiotic and biotic environments are changing, seasonal breeders respond with appropriate morphological, physiological, and behavioral adaptations in order to maximize their chances of survival and reproductive success (Bronson, 1985). Such seasonal adaptations include changes in reproductive function and behavior, frequency and magnitude of agonistic behaviors, metabolism, and immune function (Demas et al., 2010). All of these processes require substantial energy, and as energetic resources may be less plentiful during certain times of the year (e.g., winter), changes in the expression of these traits occur when energy is shifted away from certain processes and toward those that will prioritize immediate survival (Nelson and Demas, 2004). In particular, immunity is quite sensitive to the energetic state of organism, as seasonal alterations in immune function can be best predicted by changes in an animal's energetic state rather than reproductive state or photoperiodic cues (Demas, 2004). Seasonal changes in immune responses have been documented in all three branches of the immune system (i.e., innate, cell-mediated, humoral) in several different species of

mammals and birds (Martin et al., 2008c). Seasonal changes in immunity are commonly observed in inducible immune defenses because the energetic costs of mounting an immune response can be very high (e.g., can raise resting metabolic rate by as much as 50%) (Lochmiller and Deerenberg, 2000).

One of the initial and more energetically expensive immune responses is the acute phase response (APR), and the behavioral and physiological manifestations of sickness that accompany it. During the APR, pro-inflammatory cytokines are released from immune cells and act on the brain to generate the symptoms of a sickness response. Sickness responses are characterized by hyperthermia (i.e., fever) or hypothermia, anorexia, body mass loss, reductions in social, hedonic, and sexual behaviors, and hypothalamic-pituitary-adrenal (HPA) axis stimulation (Hart, 1988; Tizard, 2008). While these symptoms may appear to be a result of infection-induced weakness or malaise, these responses are actually a well-adapted mechanism to aid the host organism in clearance of the infectious agent (Hart, 1988). Blocking fever and anorexia during sickness can actually result in increased mortality via failure to eliminate the infection (Covert and Reynolds, 1977; Kluger et al., 1975; Kyriazakis et al., 1998; Vaughn et al., 1980), while blocking glucocorticoid production can cause mortality via sepsis (Bertini et al., 1988). Mounting an appropriate sickness response is clearly beneficial to an organism's survival, but being sick also carries significant energetic costs that can be detrimental to survival if too severe (Buchanan et al., 2003; Maier et al., 1994; Plata-Salaman, 1996). While variations in sickness response intensity at the extremes of the spectrum clearly negatively affect survival, the ability to modulate

sickness intensity between these “mortality endpoints” may be critical for ensuring survival in environments with variable energetic resource availability.

Seasonally breeding animals live in environments where energetic resources vary across the annual cycle (i.e., resources more plentiful in summer than winter), and studies of sickness responses in several seasonally breeding species have revealed that sickness response intensity can also vary with the seasons (reviewed in Ashley et al., 2012; Ashley and Wingfield, 2012). Collectively, the patterns of sickness response variation in these species reveal that there is not one critical season in which animals display a weak or strong sickness response, suggesting that seasonal photoperiodic cues or reproductive status may not drive variation in sickness intensity. Rather, the common predictor of sickness response intensity across these studies is the current energetic state of the animal—sickness responses are attenuated in the season in which the organism has the lowest energy reserves (i.e., lowest body mass and fat stores) (Bilbo et al., 2002; Owen-Ashley et al., 2008; Owen-Ashley et al., 2006; Prendergast et al., 2008). In further support of the hypothesis that energetic state is a predictor of sickness response intensity in seasonally breeding animals, pre-sickness body mass and body fat levels are correlated with infection-induced anorexia and body mass loss, such that animals with higher initial body masses and fat stores show greater percent decreases in food intake and body mass after LPS injection (Owen-Ashley et al., 2008; Owen-Ashley et al., 2006). These observations suggest that the magnitudes of the energetically expensive components of a sickness response are constrained

by a minimum body mass that an animal can reach before it risks its survival (Ashley and Wingfield, 2012; Owen-Ashley and Wingfield, 2007).

If energetic state is the critical predictor of seasonal variation in sickness response intensity, then the adipose hormone leptin is a promising candidate for a neuroendocrine mediator of this variation. Leptin is not only tightly coupled with the energetic state of an organism, but it also interacts with the immune system (Carlton et al., 2012; La Cava and Matarese, 2004). Leptin levels are directly proportional to the mass of adipose tissue in several mammalian species (Johnson et al., 2004; Maffei et al., 1995), and as such, high levels of leptin indicate adequate energy stores, whereas low levels are consistent with energy deficit. Leptin levels change across seasons in seasonally breeding animals, and these seasonal changes track seasonal changes in body mass and body fat (Concannon et al., 2001; Gaspar-Lopez et al., 2009; Horton et al., 2000). Immune function can be restored via leptin treatment in animals that have been food deprived or have had body fat experimentally reduced (Demas and Sakaria, 2005; Lord et al., 1998), and there is also evidence that leptin may modulate seasonal changes in immunity (Drazen et al., 2001). Although there is no yet established role of leptin in mediating seasonal variation in sickness responses, there is considerable evidence that leptin does influence sickness responses (Harden et al., 2006; Sachot et al., 2004), although the direct mechanisms and their effects are not entirely understood (Carlton et al., 2012).

The goal of the present study was to test the hypothesis that leptin serves as a neuroendocrine signal mediating seasonal variation in sickness responses. To

accomplish this, we housed male Siberian hamsters (*Phodopus sungorus*) in long and short days to induce two photoperiodic morphs, experimentally elevated leptin levels in a subset of hamsters in each morph, and then measured sickness response variables (e.g., body temperature, anorexia, body mass loss, anhedonia, nest building behavior, HPA axis activation) in response to inoculation with lipopolysaccharide (LPS), a sickness-inducing bacterial mimetic. When housed in short days, Siberian hamsters regress their gonads to a non-reproductive state and decrease food intake, body mass, and fat stores. In addition, Siberian hamsters have lower leptin levels in short days as compared to long days (Horton et al., 2000) and display less intense sickness responses (i.e., lower fever amplitude, shorter durations of and lesser decreases in food intake and body mass loss, lesser decreases in hedonic and nest shredding behaviors, higher cortisol secretion) (Bilbo et al., 2002; Bilbo et al., 2003; Wen et al., 2007). We predicted that if leptin mediates seasonal variation in sickness responses, then short-day leptin-treated hamsters would display sickness responses similar to long-day vehicle-treated hamsters and would display more intense sickness responses than short-day vehicle-treated hamsters. Leptin treatment, however, should have no effect on the sickness responses of long-day housed hamsters because previous studies in this species have shown that leptin does not enhance other measures of immunity in long-day animals even though it enhances them in short-day animals (Demas, 2002; Drazen et al., 2001).

Methods

Animals and housing conditions

Adult male (> 60 days of age) Siberian hamsters (n = 117) were obtained from our breeding colony at Indiana University. The progenitors of these animals were generously provided by Dr. Randy Nelson (Ohio State University) and Dr. Timothy Bartness (Georgia State University). In order to minimize the effects of inbreeding, our animals are outbred approximately every 10 generations. All animals were initially group housed (2-5 with same sex siblings on weaning at 17-18 days of age) in long-day photoperiods (light:dark (L:D) 16:8), and then individually housed in polypropylene cages (27.8 x 17.5 x 13.0 cm) for one week prior to the start of the experiment. Food (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO, USA) and tap water were available *ad libitum* during the entire course of the experiment. Temperature (20 ± 2°C) and humidity (50 ± 10%) were maintained at constant levels. Animals were then randomly assigned to either long (L:D 16:8) (n = 40) or short days (L:D 8:16) (n = 74) for the remainder of the study. A greater number of hamsters were housed in short days to account for reproductive non-responders (described below). All animal methods were reviewed and approved by the Institutional Animal Care and Use Committee at Indiana University Bloomington (protocol no. 10-038).

A subset of hamsters within the short-day group often fails to show reproductive responsiveness to photoperiod (i.e., do not display gonadal regression, reductions in body mass and fat stores, or changes in pelage coloration and thickness) despite prolonged exposure to short days. These individuals are referred

to as photoperiodic non-responders (Puchalski and Lynch, 1986). After ten weeks of exposure to short-day photoperiods, 36 animals were determined to be non-responders (defined by a reduction in body mass less than or equal to 10% of their mass at the beginning of the experiment) and were removed from the experiment. At the conclusion of the experiment, paired testes mass was collected to confirm short-day responsiveness (defined as a paired testes mass < 0.15 g) (Greives et al., 2008). At the end of the study, we were left with 40 hamsters exhibiting the long-day phenotype (referred to as LD from here forward) and 38 hamsters exhibiting the short-day responder phenotype (referred to as SD from here forward).

Experimental methods

During the first 10 weeks of photoperiodic treatment, hamsters were weighed weekly to the nearest 0.1 g to track photoperiodic responsiveness. After these ten weeks and when the photoperiodic non-responders were removed from the study, body mass (to the nearest 0.1 g) and food consumption (to the nearest 0.1 g) were assessed daily to establish pre-leptin treatment body mass and food intake baseline values. Food consumption was assessed by weighing the food pellets remaining in the hopper each day.

After five days, animals were surgically implanted with an osmotic mini-pump subcutaneously in the intra-scapular region under isoflurane anesthesia (Alzet 1002; 100 µl volume; 0.25 µl/h delivery rate; 14 days; Durect Corp., Cupertino, CA, USA). Half of the animals from each photoperiodic group were randomly assigned to receive mini-pumps containing recombinant murine leptin (n

= 39; 2.67 µg/µl leptin; Peprotech Inc., Rocky Hill, NJ, USA) dissolved in 0.5 M Tris buffer. The remaining animals received mini-pumps containing vehicle (n = 39; 0.5 M Tris buffer) (Drazen et al., 2001). Murine leptin has been used in several studies in Siberian hamsters and has elicited responses consistent to those reported for mice, suggesting that murine leptin can bind the Siberian hamster leptin receptor (Demas and Sakaria, 2005; Drazen et al., 2001; French et al., 2009a; Klingenspor et al., 2000). For the eight days after mini-pump implantation, body mass and food consumption were collected daily to assess surgery recovery and leptin effects on these measures.

On the eighth day after mini-pump implantation and ~15 min before the onset of darkness (long days: ~1945 h; short days: ~1545 h), a portion of the animals in each group (LD-Vehicle, n = 10; LD-Leptin, n = 10; SD-Vehicle, n = 11; SD-Leptin, n = 11) were injected intraperitoneally (i.p.) with 25 µg LPS (LPS from *Salmonella enterica* serotype typhimurium, Sigma-Aldrich, St. Louis, MO, USA; Durazzo et al., 2008; French et al., 2013) suspended in 0.1 ml sterile 0.9% saline. The remaining animals were injected i.p. with 0.1 ml sterile 0.9% saline (LD-Vehicle, n = 10; LD-Leptin, n = 10; SD-Vehicle, n = 8; SD-Leptin, n = 8). Sickness responses and behaviors (i.e., fever, anorexia, body mass loss, anhedonia, nest building) were assessed throughout the four days following injections.

Blood sampling and necropsies

Blood samples were drawn from each animal 4 h after the onset of darkness (long days: 2400 h; short days: 2000 h) 3 days before injection and on the day of

injection in order to assess circulating leptin and cortisol concentrations. We chose this time point (~4 hours after LPS injection) because it has previously been shown to be the point where there is the greatest difference in LPS-induced cortisol secretion between LD- and SD-housed Siberian hamsters (Bilbo et al., 2003). Briefly, animals were lightly anesthetized with isoflurane vapors, and blood samples were drawn from the retro-orbital sinus. Blood samples were allowed to clot at room temperature for 1 h, the clots were removed, and samples were centrifuged at 4 °C for 30 min at 2500 rpm. Serum aliquots were aspirated and stored in sealable polypropylene microcentrifuge tubes at -20 °C until assayed. All blood samples were collected within 3 min of initial handling. Animals were euthanized 5 days after LPS injection and necropsies were performed. Testes were removed, cleaned of connective tissues, and weighed to the nearest 0.1 mg in order to assess reproductive responsiveness.

Sickness response measurements

Fever, anorexia, and body mass

On the day of injection, colonic temperatures (T_c; to the nearest 0.1 °C) were collected immediately before injection and 2, 4, 6, 8, 10, 12, 16, 20, and 24 h after injection using a MicroTherma 2T thermometer (ThermoWorks, Alpine, UT, USA) and a lubricated RET-3-ISO thermocouple probe (Physitemp Instruments, Inc., Clifton, NJ, USA) inserted ~12 mm into the rectum. To assess anorexia and body mass loss, daily body mass and food consumption measurements continued until the end of the study.

Anhedonic behavior

To assess the effects of our treatments on hedonic behavior, we provided hamsters with a highly palatable sodium saccharin solution (Baillie and Prendergast, 2008). Beginning 5 days before LPS and saline injections, for the first 6 h of the dark phase (long days: 2000 h to 200 h; short days: 1600 h to 2200 h) hamsters were provided with a fluid bottle containing a solution of 0.1% sodium saccharin (saccharin sodium salt hydrate, Sigma-Aldrich, St. Louis, MO, USA) dissolved in tap water (Baillie and Prendergast, 2008). The saccharin solution bottles were weighed (to the nearest 0.1 g) before they were given and after they were collected from the hamsters each day. Presentation of saccharin solution continued daily through day 3 post-injection.

Nest building behavior

To assess the effects of our treatments on thermoregulatory behavior, beginning 5 days before LPS or saline injection, each hamster was provided with a compressed cotton nestlet weighing ~2.5 g (Ancare, Bellmore, NY, USA) for the first 6 h of the dark phase (Baillie and Prendergast, 2008). The whole nestlet was weighed (to the nearest 0.1 g) before presentation, and the unshredded portion of the nestlet was weighed after presentation. When provided a nestlet, hamsters quickly start shredding the cotton to build a nest. Nest-building is an adaptive behavior to enhance energy conservation in low temperatures, however, hamsters readily build nests in room temperature (20-23 °C) (Puchalski et al., 1988). Presentation of nestlets continued daily through day 3 post-injection.

Leptin enzyme-linked immunosorbent assay (ELISA)

Circulating leptin levels were assayed via commercially prepared mouse leptin ELISA kits (Crystal Chem, Downers Grove, IL, USA). This kit has previously been used in another non-murine rodent (Johnson et al., 2004), and prior to testing samples, we validated the assay and determined the appropriate dilutions so that leptin concentrations lay within the detectable range of the ELISA. Samples were diluted 1:4 (non-leptin treated) or 1:8 (leptin treated) with sample diluent and run in duplicate. Intra-assay variabilities were 3.9% and 5.8% for the two plates.

Cortisol enzyme immunoassay (EIA)

We assessed circulating cortisol levels to determine if our photoperiod and leptin treatments affected the magnitude of LPS-induced HPA axis activation. Cortisol is the predominant glucocorticoid in Siberian hamsters, with concentrations ~100x that of corticosterone (Reburn and Wynne-Edwards, 2000). Serum cortisol concentrations were determined in multiple enzyme immunoassays (EIAs) from a commercially prepared kit (Cortisol EIA Kit; Enzo Life Sciences, Inc., Farmingdale, NY, USA). This assay was previously validated for use in Siberian hamsters (Demas et al., 2004) and is highly specific for cortisol; cross-reactivity with corticosterone is 27.7% and < 4.0% for other steroid hormones. The sensitivity of the assay is 56.72 pg/ml. Samples were diluted to 1:80 with assay buffer and run in duplicate. Intra-assay variabilities were 6.7% and 7.4%.

Statistical analyses

All statistical tests were performed using JMP 10 (SAS Institute Inc., Cary, NC, USA), and a value of $P < 0.05$ was considered to be statistically significant. Residuals were checked for normality and homogeneity of variance, and those data that were non-normally distributed were transformed. Two LPS-treated animals were excluded from analyses (1 from SD-Leptin group; 1 from SD-Vehicle group) because they failed to exhibit any sickness symptoms. One LPS-treated animal (from SD-Leptin group) was excluded from analyses because it displayed exaggerated LPS-induced sickness symptoms and was not showing any signs of recovery by the end of the experiment (as is typical for all LPS-treated SD animals). The final sample sizes were as follows: LD-Vehicle-Saline (n=10), LD-Vehicle-LPS (n=10), LD-Leptin-Saline (n=10), LD-Leptin-LPS (n=10), SD-Vehicle-Saline (n=8), SD-Vehicle-LPS (n=10), SD-Leptin-Saline (n=8), and SD-Leptin-LPS (n=9).

Non-normally distributed variables differed in their directions and degrees of skewness, and as such, each variable was transformed with the function that best fits the data to normality. Leptin concentrations and pre-injection saccharin solution intake were log transformed, pre-injection food intake was inverse transformed, post-injection percent change in saccharin solution intake was square root transformed, and post-injection percent change in nesting material shredded was square transformed. Finally, pre-injection percent nesting material shredded was not normally distributed and could not be transformed to meet the assumptions of

normality, so a Kruskal-Wallis test was performed to determine if there were differences among groups for this measure.

Pre-injection baseline values were calculated for body mass, food intake, saccharin solution intake, and percent nesting material shredded by averaging the three daily measurements immediately prior to injections. To determine if there were effects of photoperiod or leptin treatment on pre-injection leptin levels, baseline body mass, baseline food intake, and baseline saccharin solution intake, two-way (photoperiod (2) x leptin treatment (2)) analyses of variance (ANOVA) were performed. Pre-injection body mass was included as a covariate in the model for leptin levels to control for the effect of body mass on this dependent variable. Pre-injection body mass was initially included as a covariate in the model for pre-injection food intake but was removed from the model after it was determined to not be significant predictor ($P > 0.05$).

Changes in colonic temperature across groups and time were assessed via Linear Mixed Models (LMM) with leptin treatment, injection, time, and their interactions as fixed effects and animal ID as a random effect. Due to natural circadian variation in temperature between LD and SD hamsters, individual LMMs were run for each photoperiod. We used a LMM for this measure, as opposed to a repeated-measures ANOVA, because the thermometer failed during the temperature collection for six animals at the 4 h time-point, and the LMM allowed us to keep these animals in our analyses. Pairwise comparisons were conducted using *t*-tests between each LPS-injected group and their respective saline control at each time point.

Because photoperiod affected pre-injection body mass, food intake, and saccharin solution intake (see Results), post-injection changes in these measurements were expressed as percentages of each animal's baseline values for these variables. Although nest shredding was not affected by photoperiod, for continuity in presentation of results, this measurement was also expressed as a percentage of baseline. Differences in these repeated measures (i.e., percent change in food intake, percent change in body mass, percent change in saccharin solution intake, and percent change in nesting material shredded) were assessed via repeated-measures ANOVAs with photoperiod, leptin treatment, injection, and their interactions as between subjects variables and time and its interactions as the within-subjects variables. The within-subject comparisons for percent change in body mass, percent change in saccharin solution intake, and percent change in nesting material shredded violated the assumptions of sphericity and were Greenhouse-Geisser (GG)-corrected. Differences in serum cortisol concentrations among the groups were assessed with a three-way (photoperiod (2) x leptin treatment (2) x injection (2)) ANOVA. Post-hoc comparisons between pairwise means were conducted using Tukey's honestly significant difference (HSD) tests when the overall ANOVA was significant. Effect size estimates for ANOVAs and pair-wise comparisons were calculated by eta squared (η^2) and Cohen's d, respectively.

Results

Pre-injection/Post-leptin baseline measures

Leptin levels in both vehicle- and leptin-treated animals were related to the animal's pre-injection body mass ($F_{1,70} = 22.26$, $P < 0.001$, $\eta^2 = 0.116$). In addition, leptin levels were affected by photoperiod ($F_{1,70} = 5.67$, $P = 0.02$, $\eta^2 = 0.029$), leptin treatment ($F_{1,70} = 82.77$, $P < 0.001$, $\eta^2 = 0.430$) and the photoperiod x leptin interaction ($F_{1,70} = 12.01$, $P < 0.001$, $\eta^2 = 0.062$) (Table 1). Specifically, SD-Leptin hamsters showed leptin levels that were higher than the levels of SD-Vehicle hamsters ($P < 0.01$, d = 2.567) but statistically equivalent to LD-Vehicle levels ($P > 0.90$, d = 0.181).

Prior to injection, photoperiod affected baseline body mass ($F_{1,71} = 92.49$, $P < 0.001$, $\eta^2 = 0.548$), such that SD hamsters had significantly lower body masses than LD hamsters; however, neither leptin treatment ($F_{1,71} = 1.53$, $P = 0.220$, $\eta^2 = 0.009$) or the photoperiod x leptin interaction ($F_{1,71} = 3.85$, $P = 0.0538$, $\eta^2 = 0.023$) had effects on pre-injection body mass (Table 1). Additionally, pre-injection baseline food intake was affected by both photoperiod ($F_{1,71} = 39.09$, $P < 0.001$, $\eta^2 = 0.320$) and leptin treatment ($F_{1,71} = 8.21$, $P = 0.006$, $\eta^2 = 0.067$) and a trend toward the photoperiod x leptin interaction ($F_{1,71} =$, 3.94, $P = 0.051$, $\eta^2 = 0.0322$) (Table 1). Specifically, SD hamsters consumed less food per day than LD hamsters ($T = 6.25$, $P < 0.001$, d = -1.3471), and leptin-treated hamsters consumed less food per day than vehicle-treated hamsters ($T = 2.86$, $P = 0.003$, d = -0.490); however, the effect of leptin on food intake was largely driven by SD-Leptin hamsters exhibiting lower daily food consumption than SD-Vehicle hamsters.

Pre-injection baseline saccharin solution intake was affected by photoperiod ($F_{1,70}$ = 9.17, P = 0.003, η^2 = 0.116) and the photoperiod x leptin interaction ($F_{1,70}$ = 5.38, P = 0.023, η^2 = 0.069) but not leptin treatment alone ($F_{1,70}$ = 0.004, P = 0.953, η^2 = 0.0004) (Table 1). Specifically, SD-Leptin hamsters consumed less saccharin solution than LD-Leptin hamsters. Pre-injection baseline nest shredding was not affected by our treatments, and percent nesting material shredded did not differ among groups (H = 3.97, P = 0.265) (Table 1).

Table 1. Mean (± SEM) circulating leptin concentration, body mass, food intake, saccharin solution intake, and percent nesting material shredded prior to LPS and saline injections in long-day (LD) and short-day (SD) housed male Siberian hamsters implanted with vehicle- or leptin-filled osmotic mini-pumps. Baseline measurements were calculated by averaging the values collected for the measurements during the 3 days prior to LPS or saline injection. Groups with different letters indicate statistically significant differences between group means ($P < 0.05$); groups sharing the same letter are statistically equivalent.

	Vehicle	Leptin
Leptin Concentration (ng / ml)		
LD	12.03 ± 1.08[a]	24.65 ± 2.54[c]
SD	4.06 ± 0.53[b]	12.90 ± 1.30[a]
Baseline Body Mass (g)		
LD	41.4 ± 1.2[a]	44..9 ± 1.0[a]
SD	33.2 ± 1.1[b]	32.4 ± 1.1[b]
Baseline Food Intake (g / day)		
LD	5.3 ± 0.2[a]	5.1 ± 0.1[a,b]
SD	4.5 ± 0.2[b]	3.9 ± 0.2[c]
Baseline Saccharin Solution Intake (g / 6 h)		
LD	2.1 ± 0.2[a,b]	2.5 ± 0.2[a]
SD	1.9 ± 0.2[a,b]	1.5 ± 0.2[b]
Baseline Percent Nesting Material Shredded		
LD	87.4 ± 4.5	97.2 ± 1.2
SD	92.0 ± 4.7	97.5 ± 1.8

Febrile/Hypothermic responses

Within each photoperiod, colonic temperature was significantly affected by injection (LD: $F_{1, 36}$ = 9.76, P = 0.004; SD: $F_{1, 31.11}$ = 7.667, P = 0.009), time (LD: $F_{9, 324}$ = 68.42, P < 0.001; SD: $F_{9, 273.2}$ = 63.02, P < 0.001), and the interaction between injection and time (LD: $F_{9, 324}$ = 14.06, P < 0.001; SD: $F_{9, 273.2}$ = 22.21, P < 0.001) (Figure 1). In addition, the pattern of colonic temperature was influenced by the leptin x time interaction in LD animals only ($F_{9, 324}$ = 3.06, P = 0.002). Colonic temperature was not predicted by leptin treatment or any of the additional interaction terms in either photoperiods (P > 0.05 in all cases). LPS-injected LD-Vehicle, SD-Vehicle, and SD-Leptin hamsters had significantly higher T_c at 2 hours after injections as compared to their respective saline-injected controls (T > 3.06, P < 0.05 in all cases, $d_{LD\text{-}Veh}$ = 1.374, $d_{SD\text{-}Veh}$ = 2.408, $d_{SD\text{-}Lep}$ = 1.837). In the LPS-injected SD-Vehicle and SD-Leptin hamsters, the higher temperature in animals appears to be due to a transient hypothermic response in the saline-injected controls, rather than an increase in temperature in LPS-treated animals. After these higher temperatures at 2 hours post-LPS, all LPS-treated hamsters showed varying degrees of hypothermia. Specifically, LD-Vehicle, LD-Leptin, and SD-Leptin hamsters each showed hypothermia at two or three time points post-LPS treatment (LD-Vehicle: 6h, 24h; Figure 1A; LD-Leptin: 6h, 12h, 24h, Figure 1B; SD-Leptin: 12h, 16h; Figure 1B) as compared to their respective saline-injected controls (T > 2.60, P < 0.05 in all cases, LD-Veh: d_{6h} = -2.634, d_{24h} = -1.644; LD-Lep: d_{6h} = -2.2637, d_{12h} = -1.814, d_{24h} = -1.170; SD-Lep: d_{12h} = -1.493, d_{16h} = -1.34). In contrast, SD-Vehicle hamsters showed hypothermia at all measured time points from 6 h to 24

h post-LPS treatment as compared to their respective saline-injected controls (T > 2.31, P < 0.05 in all cases, d < -1.048; Figure 1A).

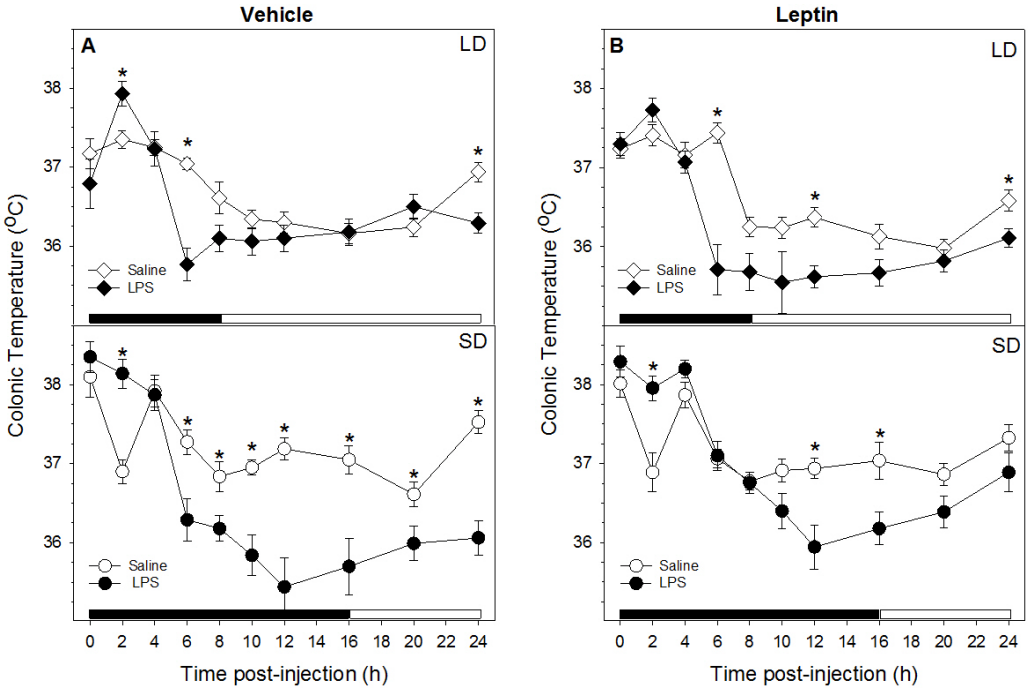

Figure 1. Mean (± SEM) colonic temperature following LPS and saline treatments delivered at the 0 h time point in long-day (LD; top panels) and short-day (SD; bottom panels) housed male Siberian hamsters implanted with (A) vehicle-filled and (B) leptin-filled osmotic mini-pumps. Black and white bars at the bottom of the graphs indicate the active, dark (black) and inactive, light (white) phases of the light-dark cycle for each photoperiodic morph. Within each panel: $^{*}P < 0.05$ versus saline-treated group exposed to the same photoperiod and osmotic mini-pump treatment.

Anorexia

Changes in food intake after injections were affected by photoperiod ($F_{1,67}$ = 20.15, P < 0.001, η^2 = 0.083), injection ($F_{1,67}$ = 132.55, P < 0.001, η^2 = 0.548), and the photoperiod x injection interaction ($F_{1,67}$ = 16.06, P < 0.001, η^2 = 0.066), such that SD hamsters from both the vehicle- and leptin-treated groups showed decreased magnitudes of LPS-induced anorexia as compared to LD hamsters

(Figure 2). In addition, food intake changed across the course of the four days post injection (within subjects, $F_{3,65} = 122.78$, $P < 0.001$, $\eta^2 = 0.531$). Additional within subjects analyses revealed time x injection ($F_{3,65} = 50.51$, $P < 0.001$, $\eta^2 = 0.206$), time x photoperiod x injection ($F_{3,65} = 3.24$, $P = 0.028$, $\eta^2 = 0.011$), and time x photoperiod x leptin interactions ($F_{3,65} = 4.53$, $P = 0.006$, $\eta^2 = 0.017$). Specifically, LPS-treated LD-Vehicle hamsters showed decreased food intake on days 1, 2, 3, and 4 post-LPS treatment as compared to their respective saline treated controls ($P < 0.03$ for all comparisons, $d_{day1} = -4.931$, $d_{day2} = -3.587$, $d_{day3} = -1.989$, $d_{day4} = -1.799$), while LPS-treated SD-Vehicle hamsters showed decreased food intake on days 1 and 2 post-LPS as compared to controls ($P < 0.01$ for day 1 and 2 comparisons, $d_{day1} = -2.727$, $d_{day2} = -1.632$; $P > 0.90$ for day 3 and 4 comparisons, $d_{day3} = -0.356$, $d_{day4} = 0.498$; Figure 2A). Alternatively, leptin-treated hamsters from both photoperiods showed attenuated LPS-induced anorexic responses as compared to vehicle-treated hamsters. Specifically, LPS-treated LD-Leptin hamsters only showed decreased food intake on days 1, 2, and 3 post-injection as compared to their respective saline-treated controls ($P < 0.01$ for day 1, 2, and 3 comparisons, $d_{day1} = -9.349$, $d_{day2} = -4.324$, $d_{day3} = -2.07$; $P > 0.08$ for day 4 comparison, $d_{day4} = -1.3149$), while LPS-treated SD-Leptin hamsters showed decreased food intake only on day 1 post-injection as compared to controls ($P < 0.05$ for day 1 comparison, $d_{day1} = -2.701$; $P > 0.80$ for day 2, 3, and 4 comparisons, $d_{day2} = -0.541$, $d_{day3} = -0.216$, $d_{day4} = 0.425$; Figure 2B).

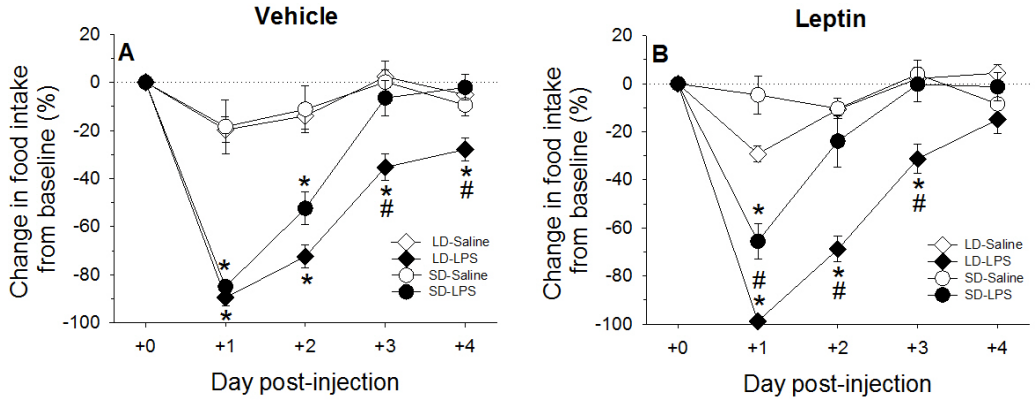

Figure 2. Mean (± SEM) percent change in daily food intake from baseline following LPS and saline treatment delivered on day 0 in long-day (LD) and short-day (SD) housed male Siberian hamsters implanted with (A) vehicle-filled and (B) leptin-filled osmotic mini-pumps. Day +1 represents the time period from 0-24 h after LPS or saline injection, Day +2 represents the time period from 24-48 h after LPS or saline injection, and so forth. $^{*}P < 0.05$ versus saline-treated group exposed to the same photoperiod and osmotic mini-pump treatment. $^{\#}P < 0.05$ versus SD LPS-treated group exposed to the same osmotic mini-pump treatment.

Body mass loss

Changes in body mass after injections were affected by injection ($F_{1,67}$ = 130.30, $P < 0.001$, $\eta^2 = 0.631$) and the leptin x injection interaction ($F_{1,67} = 5.04$, $P = 0.0281$, $\eta^2 = 0.024$) (Figure 3). Percent body mass loss changed across the course of the four days post injection (within subjects, $F_{2.03,136.31} = 30.75$, $P < 0.001$, G-G corrected, $\eta^2 = 0.190$). In addition, within subjects analyses revealed time x photoperiod ($F_{2.03,136.31} = 26.20$, $P < 0.001$, G-G corrected, $\eta^2 = 0.162$), time x injection ($F_{2.03,136.31} = 10.05$, $P < 0.001$, G-G corrected, $\eta^2 = 0.062$), and time x photoperiod x injection interactions ($F_{2.03,136.31} = 25.14$, G-G corrected, $P < 0.001$, $\eta^2 = 0.156$). Specifically, LPS-treated LD-Vehicle and SD-Vehicle hamsters showed body mass decreases that were greater than their respective saline-treated controls at all 4 days post-injection ($P < 0.03$ for all comparisons, LD: d_{day1} = -

2.686, d_{day2} = -4.087, d_{day3} = -3.596, d_{day4} = -3.409; SD: d_{day1} = -2.823, d_{day2} = -3.554, d_{day3} = -2.871, d_{day4} = -1.755; Figure 3A), although body mass appeared to be recovering toward baseline in SD-Vehicle hamsters by day 4. LPS-treated LD-Leptin hamsters only showed post-injection body mass decreases that were greater than their respective saline-treated controls at days 2, 3, and 4 post-injection (P < 0.001 for day 2, 3 and 4 comparisons, d_{day2} = -1.421, d_{day3} = -1.721, d_{day4} = -1.702; P > 0.17 for day 1 comparison, d_{day1} = -1.176; Figure 3B). Alternatively, LPS-treated SD-Leptin hamsters showed post-injection body mass decreases that were greater than their respective saline-treated controls only at days 1, 2, and 3 post-injection (P < 0.001 for day 1, 2 and 3 comparisons, d_{day1} = -2.898, d_{day2} = -2.917, d_{day3} = -2.907), while the LPS-induced body mass loss did not persist to day 4 post-injection (P > 0.57, d_{day4} = -1.108; Figure 3B).

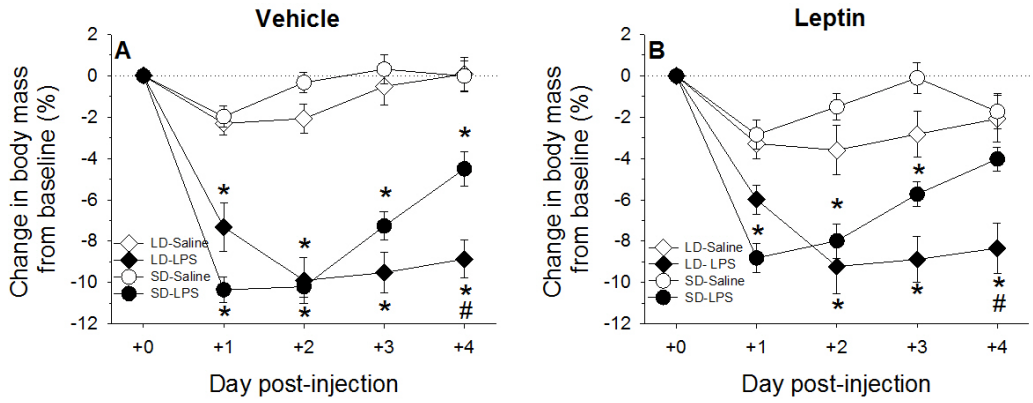

Figure 3. Mean (± SEM) percent change in body mass from baseline following LPS and saline treatment delivered on day 0 in long-day (LD) and short-day (SD) housed male Siberian hamsters implanted with (A) vehicle-filled and (B) leptin-filled osmotic mini-pumps. Day +1 represents the time period from 0-24 h after LPS or saline injection, Day +2 represents the time period from 24-48 h after LPS or saline injection, and so forth. $^{*}P$ < 0.05 versus saline-treated group exposed to the same photoperiod and osmotic mini-pump treatment. $^{\#}P$ < 0.05 versus SD LPS-treated group exposed to the same osmotic mini-pump treatment.

Saccharin solution intake

Changes in saccharin solution intake after injections were affected by photoperiod ($F_{1,66}$ = 18.62, P < 0.001, η^2 = 0.184), injection ($F_{1,66}$ = 5.41, P = 0.023, η^2 = 0.054), and the photoperiod x injection interaction ($F_{1,67}$ = 4.47, P = 0.038, η^2 = 0.044) (Figure 4). Additionally, percent saccharin intake changed across time (within subjects, $F_{2.47,163.01}$ = 3.39, P = 0.027, G-G corrected, η^2 = 0.043) and was affected by the time x photoperiod x leptin interaction (within subjects, $F_{2.47,163.01}$ = 5.17, P = 0.004, G-G corrected, η^2 = 0.065). As there was a lot of variance in this measure, post hoc analyses only revealed that, at the 48-54 h time point, LPS-treated LD-Vehicle hamsters showed a greater percent decrease in saccharin solution intake as compared to LPS-treated SD-Vehicle hamsters (P < 0.01, d = 1.562; Figure 4A). However, LPS-treated hamsters (independent of photoperiod and leptin treatments) showed greater percent decreases in saccharin solution at the 0-6 h time point as compared to saline-treated hamsters (T = 2.18, P < 0.04, d = -0.532).

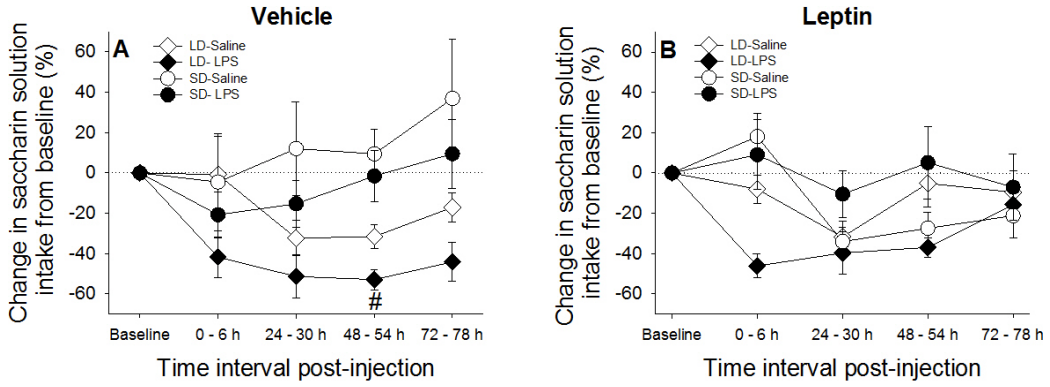

Figure 4. Mean (± SEM) percent change in saccharin solution intake from baseline following LPS and saline treatment delivered at the 0 h time point in long-day (LD) and short-day (SD) housed male Siberian hamsters implanted with (A) vehicle-filled and (B) leptin-filled osmotic mini-pumps. $^{\#}P$ < 0.05 versus SD LPS-treated group exposed to the same osmotic mini-pump treatment.

Nest building behavior

Percent changes in nesting material shredded were affected by injection ($F_{1,67}$ = 118.51, $P < 0.001$, $\eta^2 = 0.626$), but not by photoperiod, leptin, or any of the interaction terms ($P > 0.05$ for all effects) (Figure 5). In addition, percent nesting material shredded changed over time ($F_{2.7,183.4}$ = 46.63, $P < 0.001$, G-G corrected, $\eta^2 = 0.270$) and changed according to the time x injection interaction ($F_{0.9,52.3}$ = 52.32, $P < 0.001$, G-G corrected, $\eta^2 = 0.303$). LPS-treated LD-Vehicle, SD-Vehicle, and SD-Leptin hamsters showed greater decreases in nesting material shredded as compared to saline-injected controls at the 0-6 h and 24-30 h time points ($P < 0.02$ for all comparisons, LD-Veh: $d_{0\text{-}6h}$ = -1.383, $d_{24\text{-}30h}$ = -4.933; SD-Veh: $d_{0\text{-}6h}$ = -1.684, $d_{24\text{-}30h}$ = -4.306; SD-Lep: $d_{0\text{-}6h}$ = -2.830, $d_{24\text{-}30h}$ = -4.416; Figures 5A and 5B). Nest building behavior in these groups had recovered to levels equivalent to saline-injected controls by the 48-54 h time point. LPS-treated LD-Leptin hamsters showed greater percent decreases in nesting material shredded as compared to saline-injected controls at the 24-30 h and the 48-54 h time points ($P < 0.02$, $d_{24\text{-}30h}$ = -23.403, $d_{48\text{-}54h}$ = -1.721; Figure 5B), and nest building behavior had recovered in this group by the 72-78 hour time point.

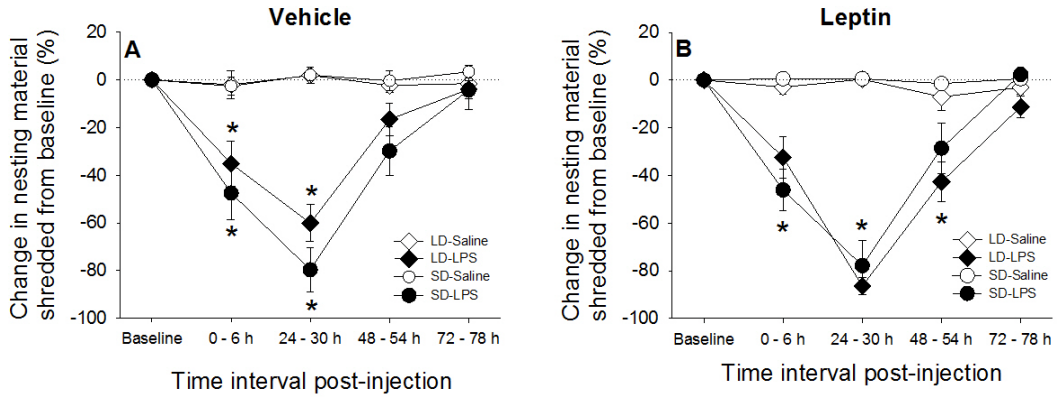

Figure 5. Mean (± SEM) percent change in nesting material shredded from baseline following LPS and saline treatment delivered at the 0 h time point in long-day (LD) and short-day (SD) housed male Siberian hamsters implanted with (A) vehicle-filled and (B) leptin-filled osmotic mini-pumps. $^{*}P < 0.05$ versus saline-treated group exposed to the same photoperiod and osmotic mini-pump treatment.

Serum cortisol

Post-injection circulating cortisol levels were affected by photoperiod ($F_{1,67} = 19.55$, $P < 0.001$, $\eta^2 = 0.148$) and injection ($F_{1,67} = 41.73$, $P < 0.001$, $\eta^2 = 0.317$) (Figure 6). Specifically, SD hamsters had higher levels of circulating cortisol than LD hamsters ($T = 4.42$, $P < 0.01$, d = 0.880), and LPS-treated hamsters had higher cortisol levels than saline-treated hamsters ($T = 6.46$, $P < 0.01$, d = 1.377).

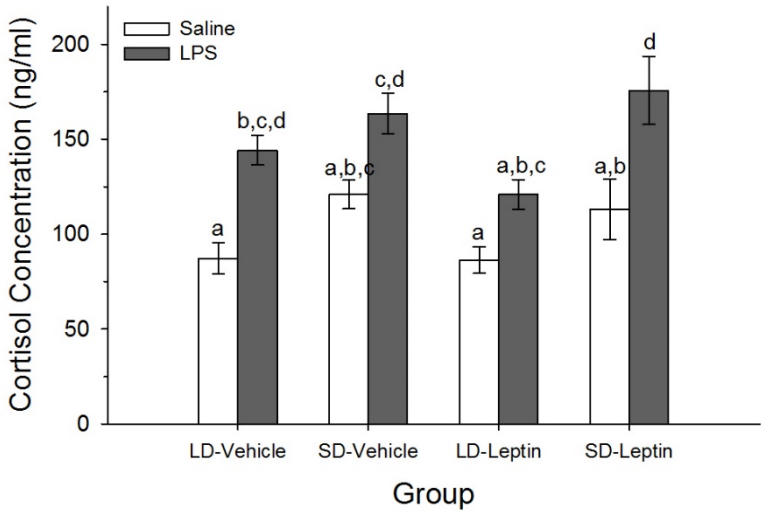

Figure 6. Mean (± SEM) circulating serum cortisol taken 4 h following LPS and saline treatments in long-day (LD) and short-day (SD) housed male Siberian hamsters implanted with vehicle-filled or leptin-filled osmotic mini-pumps. Groups with different letters indicate statistically significant differences between group means ($P < 0.05$); groups sharing the same letter are statistically equivalent.

Discussion

Our findings demonstrate that leptin acts as a neuroendocrine regulator of sickness. Specifically, leptin mediated some but not all symptoms of seasonal sickness response variation in Siberian hamsters. In this experiment, we experimentally elevated the circulating leptin levels of short-day hamsters so that they were comparable to the leptin levels of long-day hamsters and then assessed various components of their sickness responses to LPS. Although animals showed mainly hypothermic responses to LPS, short-day leptin-treated hamsters showed patterns of LPS-induced hypothermia that were greatly attenuated as compared to short-day vehicle-treated hamsters. The attenuated pattern of LPS-induced hypothermia in short-day leptin-treated hamsters was more similar to the

hypothermic responses exhibited by long-day vehicle- and long-day leptin-treated hamsters. Conversely, while we predicted that short-day leptin-treated hamsters would show longer durations and greater magnitudes of LPS-induced anorexia and body mass loss as compared to short-day vehicle-treated hamsters, we actually found that short-day leptin-treated hamsters had the shortest durations and lowest magnitudes of LPS-induced anorexia and body mass loss of all four groups. Finally, our measures of anhedonic behavior (i.e., saccharin solution intake), thermoregulatory behavior (i.e., nesting material shredding), and circulating cortisol were affected by LPS injection. LPS injection resulted in decreased saccharin solution intake and nest material shredding and increased circulating cortisol levels, however, none of the LPS-induced decreases and increases in these measures were modulated by leptin treatment.

Collectively, these results suggest that leptin acts as a mediator of seasonally appropriate fever/hypothermic responses. Although we did not initially predict that animals would show hypothermic responses to LPS, hypothermic responses to LPS are not uncommon and have been observed in several species of birds and mammals in experimental settings where a hypothermic response was not predicted (Burness et al., 2010; Martin et al., 2008b; Owen-Ashley et al., 2008). Hypothermic responses may be beneficial as they can help improve survival chances during severe systemic inflammation and sepsis (Romanovsky and Szekely, 1998). Additionally, hypothermia may be an adaptive strategy used for fighting infection when energy levels are low, as fever can only be beneficial if its heightened energy demands can be supported (Romanovsky and Szekely, 1998). In

support of this hypothesis, green iguanas (*Iguana iguana*) can show both febrile and hypothermic responses to LPS, and the type of response that a lizard displays is dependent on its body mass (Deen and Hutchison, 2001). Heavier lizards choose to behaviorally thermoregulate to higher body temperatures (i.e., fever), while smaller lizards choose to behaviorally thermoregulate to lower body temperatures (i.e., hypothermia) after LPS-injection (Deen and Hutchison, 2001). Further support for this hypothesis comes from studies showing that negative energy balance, induced via fasting, results in attenuated fever responses to LPS in rats and hamsters (Bilbo and Nelson, 2002; Inoue and Luheshi, 2010; Inoue et al., 2008). There is also evidence that circulating leptin levels play a role in fever and hypothermic responses to LPS (Steiner and Romanovsky, 2007), as neutralizing leptin via a leptin anti-serum depresses or abolishes fever in rats treated with LPS (Harden et al., 2006; Sachot et al., 2004). Leptin receptor-deficient rats (Koletsky *f/f*) show increased durations of LPS-induced hypothermia as compared to rats with functional receptors (Koletsky *F/?*) (Steiner et al., 2004); however, these fever- and hypothermia-modulating effects of leptin are not always seen in all experimental systems or at all LPS doses (Inoue and Luheshi, 2010; Ivanov and Romanovsky, 2002). Therefore, it is possible that the short-day vehicle-treated hamsters showed the greatest magnitude of LPS-induced hypothermia because they had the lowest energy stores and leptin levels and were engaging in energy conservation. Additionally, increased circulating leptin levels in short-day leptin-treated hamsters may have acted as a signal of increased energy stores in these animals and mediated the attenuated hypothermic response.

We did not expect that long-day hamsters would show LPS-induced hypothermia, as they were not energetically challenged and because other studies in this species have shown that both long-day and short-day hamsters exhibit febrile responses to LPS (Bilbo et al., 2002; Wen et al., 2007). Although we used the same dose of LPS in our study as was used in the previous studies, this difference in our results from those of the other studies may be due to differences in the species and serotypes of the LPS used in these studies. In the current study, we used LPS from *Salmonella enterica*, whereas LPS from *Escherichia coli* was used in other studies. Different serotypes of *E. coli* can produce different patterns of LPS-induced fever and hypothermia (Akarsu and Mamuk, 2007); therefore, it seems likely that LPS from different species could also elicit different fever and hypothermia patterns. We have previously used LPS from *S. enterica* in our lab and found that it induced hypothermia, rather than fever in the hamsters (French et al., 2013a). These species differences in LPS may also explain why we did not see photoperiodic differences in LPS-induced decreases in nest building behavior as previous studies have reported (Prendergast et al., 2008; Wen et al., 2007). For instance, huddling in a nest would likely prevent radiative heat loss and reduce hypothermia, so animals exhibiting sickness-induced hypothermia may want to avoid nesting during illness. As such, if sickness-induced hypothermia is adaptive in this experimental context, then we would not expect to see short-day attenuation of LPS-induced decreases in nest building behavior. However, as we still saw seasonal variation in sickness responses between the long-day and short-day groups in several of our measures (i.e., temperature, anorexia, body mass loss, cortisol levels), these LPS species

differences should not influence our ability to make meaningful comparisons to previous studies.

One of our initial predictions was that if leptin mediated seasonal changes in sickness responses, then we expected that providing short-day hamsters with supplemental leptin would increase the magnitude and duration of LPS-induced anorexia and body mass loss. Rather, we found that leptin treatment attenuated these measures in short-day hamsters. Leptin was first characterized for its role in regulating food intake and body mass, with experimentally increased leptin levels resulting in decreases in food intake and body mass (Friedman and Halaas, 1998). Furthermore, circulating leptin levels are increased in response to LPS injection (Sarraf et al., 1997). Because LPS increases circulating leptin levels, and increased leptin levels decrease food intake and body mass, it seems that leptin could be a likely candidate for regulating infection-induced anorexia and body mass loss; however, the evidence supporting this relationship is mixed (Carlton et al., 2012). Our results suggest that leptin modulates LPS-induced anorexia and body mass loss because these measures are attenuated in both the long-day and short-day leptin treated groups; however, as it does not regulate these responses in our predicted manner, it is likely not regulating these responses in this seasonal context.

Another possible explanation for the unexpected attenuation of LPS-induced anorexia and body mass loss in the short-day leptin-treated group is that anorexia and body mass loss were attenuated in this group to counteract the effects of the weakened hypothermic response. If reducing hypothermia resulted in greater energy expenditure, then these animals might have had to preserve energy by

reducing anorexia in order to avoid reaching a level of survival-risking negative energy balance (Ashley and Wingfield, 2012; Owen-Ashley and Wingfield, 2007). If reductions in LPS-induced anorexia in short-day leptin-treated animals are the result of an energy conservation mechanism, then it is possible that seasonal changes in infection-induced fever/hypothermia are regulated by circulating leptin levels while changes in infection-induced anorexia are regulated by some other energetic mechanism. There are several other orexigenic and anorexigenic hormones (e.g., ghrelin, neuropeptide Y, alpha-melanocyte-stimulating hormone, cholecystokinin, corticotropin-releasing hormone) that have been experimentally linked to infection-induced anorexia (Carlton et al., 2012). Therefore, it is likely that leptin may be just one among many hormones that act to coordinate seasonally appropriate sickness.

Further support of this idea that different symptoms of seasonal sickness responses are regulated by different hormonal and physiological mechanisms comes from previous studies that have examined the roles of other seasonally variable traits in sickness response modulation (Adelman and Martin, 2009). For example, removing the endogenous melatonin signal that organizes the seasonal response in Siberian hamsters (i.e., the short-day morph is induced when animals secrete melatonin for long durations; the long-day morph is induced when animals secrete melatonin for short durations) via pinealectomy causes hamsters housed in short days to display long-day-like patterns of LPS-induced anorexia, body mass loss, and reductions in nesting material use (Wen et al., 2007). Providing short-day pinealectomized hamsters with peripheral melatonin implants restores short-day-

typical responses for these three LPS-induced measures (Freeman et al., 2007). Surprisingly, short-day pinealectomized animals still show short-day-typical fever responses (Wen et al., 2007), suggesting that this response is mediated by some factor other than melatonin. Long-day hamsters that are castrated show shortened durations of LPS-induced anorexia and decreased magnitudes of LPS-induced body mass loss as compared to uncastrated long-day controls; however, castration results in no attenuations in LPS-induced decreases in nesting material use (Prendergast et al., 2008).

While these studies show that manipulating different seasonally variable traits (e.g., photoperiodic rhythms, melatonin, testosterone) affects some of the same and some different components of sickness responses, manipulating these traits also have effects on the animal's body mass. Pinealectomy results in short-day hamsters displaying long-day-like body masses, while providing peripheral melatonin to these pinealectomized animals once again renders their body masses short-day-like (Freeman et al., 2007; Wen et al., 2007). Additionally, castrating long-day hamsters results in body mass decreases (Prendergast et al., 2008). Even though our study suggests that leptin does not influence all components of seasonal sickness response variation, we cannot eliminate the hypothesis that energetic state is a primary driver of the seasonal differences in any of these symptoms of sickness.

Because we did not manipulate actual body mass in this study (and leptin is only one among many signals of energy) and prior studies have not controlled for changes in body mass while manipulating other seasonally-changing variables, there could be other energetic hormones modulating these responses. Additional

support for the need to continue to test the hypothesis that energetic state drives seasonal variation in sickness responses comes from observations that these correlations between sickness responses and body mass extended beyond seasonal breeders. For instance, in rats, ranging from lean to obese, there are positive correlations between body mass and the length of time it takes for an animal to recover from LPS-induced sickness (i.e., heavier animals take longer to recover) and the number of sickness symptoms that an animal displays (i.e., heavier animals display more sickness symptoms) (Pohl et al., 2014). Exploring the connections between other energetic hormones and seasonal variation in sickness responses will hopefully allow us to narrow in on the mechanisms promoting these correlations between body mass and sickness response intensity in seasonally breeding animals, as well as non-seasonal model organisms. We may also gain a better understanding of the mechanisms driving these relationships between energy stores and sickness responses by investigating the role of cytokines as intermediate factors between energy and sickness. Leptin (and other seasonal and energetic factors) is likely driving seasonal differences in sickness response intensity through effects on cytokine release and sensitivity (Dantzer, 2004; Zimmerman et al., 2014). Previous studies have shown photoperiodic differences in cytokine release (Bilbo et al., 2002), gene expression (Pyter et al., 2005), and sensitivity (Wen and Prendergast, 2007), so the effects of seasonal leptin variation on these factors should be a target of future investigations.

In conclusion, our data show that leptin does play a role in regulating seasonal variation in sickness response symptoms. Although leptin may not

influence all the components of sickness responses in Siberian hamsters, leptin supplementation clearly attenuated the hypothermic response to LPS in short-day hamsters. This leptin-induced suppression of hypothermia in short-day hamsters may have required animals to conserve energy by reducing their displays of LPS-induced anorexia. Our results (and the results of previous studies) do not exclude the hypothesis that seasonal variation in sickness responses is a product of seasonal changes in energetic stores. Instead, our results suggest that individual symptoms in the sickness responses of these seasonally breeding animals may be modulated by various energetic and non-energetic hormones, providing opportunities for future investigations.

CHAPTER 3: Body mass affects seasonal variation in sickness intensity in a seasonally-breeding rodent

Data published in: Carlton, E.D. and Demas, G.E. 2015. Body mass affects seasonal variation in sickness intensity in a seasonally-breeding rodent. *Journal of Experimental Biology, in press.*

Abstract

Species that display seasonal variation in sickness intensity show the most intense response in the season during which they have the highest body mass, suggesting that sickness intensity may be limited by an animal's energy stores. Siberian hamsters (*Phodopus sungorus*) display lower body masses and less intense sickness when housed in short, winter-like days as opposed to long, summer-like days. To determine if reduced sickness intensity displayed by short-day hamsters is a product of seasonal changes in body mass, we food-restricted long-day hamsters so that they exhibited body mass loss that mimicked the natural photoperiod-induced loss of body mass in short-day hamsters. We then experimentally induced sickness with lipopolysaccharide (LPS) and compared sickness responses among long-day food restricted and long- and short-day *ad libitum* fed groups, predicting that long-day restricted hamsters would show sickness responses comparable to short-day *ad libitum* hamsters and attenuated in comparison to long-day *ad libitum* hamsters. We found that long-day restricted hamsters showed attenuated LPS-induced anorexia, loss of body mass, and hypothermia compared to long-day *ad libitum* animals; however, anorexia was still more pronounced than in short-day *ad libitum* animals. Additionally, LPS-induced anhedonia and decreases in nest building were not influenced by body mass. Results of hormone assays suggest that cortisol levels could play a role in the attenuation of sickness in long-day restricted

hamsters, indicating that future research should target the roles of glucocorticoids and natural variation in energy stores in seasonal sickness variation.

Introduction

Mounting an appropriate immune response to infection is a necessity for survival in a pathogen-rich environment. An organism's immune response is sensitive to both its external and internal environments and can be influenced by variables such as environmental temperature, photoperiod, stress, reproductive effort, and energy stores (Demas and Nelson, 1998; Lochmiller and Deerenberg, 2000; Martin et al., 2008c; Sheldon and Verhulst, 1996). While each of these variables can fluctuate individually across short time spans, for seasonally breeding animals, these variables often change simultaneously with transitions between seasons. Therefore, one challenge for determining the specific physiological and environmental variables underlying seasonal changes in immunity is disentangling the effects of each of these variables from one another.

The acute phase response (APR) is one component of the immune system that can vary seasonally (Adelman and Martin, 2009; Ashley and Wingfield, 2012). When the APR is activated at an infection site, neutrophils and macrophages release pro-inflammatory cytokines which not only facilitate the recruitment of other immune cells to this local site of infection, but also act on the brain to generate sickness responses. Sickness responses are characterized by fever or hypothermia, anorexia, body mass loss, reductions in social and hedonic behaviors, and hypothalamic-pituitary-adrenal (HPA) endocrine axis activation (Hart, 1988;

Tizard, 2008). These sickness symptoms are generated as adaptive mechanisms for fighting off infection (Hart, 1988), and in several seasonally breeding animals, sickness intensity varies across the seasons (Bilbo et al., 2002; Owen-Ashley et al., 2008; Owen-Ashley et al., 2006; Owen-Ashley and Wingfield, 2006). Because generating a sickness response is critical for eliminating pathogens, variation in the magnitude of this response can profoundly affect an animal's ability to survive an infection (Kluger et al., 1975; Murray and Murray, 1979; Vaughn et al., 1980). Thus, questions remain: why and how are seasonally breeding animals modulating sickness intensity?

When looking across species in which seasonally variable sickness responses have been documented, the common variable that predicts which season an animal will show reduced or enhanced sickness is the animal's body mass, not reproductive state or day length (Ashley and Wingfield, 2012; Carlton et al., 2012). Specifically, seasonally breeding animals show attenuated sickness responses to the bacterial mimetic lipopolysaccharide (LPS) in the season that they have the lowest body masses (Bilbo et al., 2002; Owen-Ashley et al., 2008; Owen-Ashley et al., 2006; Owen-Ashley and Wingfield, 2006). Siberian hamsters (*Phodopus sungorus*) are one species that displays seasonal variation in sickness intensity. Hamsters display more intense sickness responses (i.e., greater fever amplitude, longer durations of and greater decreases in food intake and body mass loss, greater decreases in hedonic and nest building behaviors) to LPS when housed in long, summer-like photoperiods as compared to short, winter-like photoperiods (Bilbo et al., 2002; Wen et al., 2007; Wen and Prendergast, 2007). Hamsters housed in long

photoperiods remain reproductively active and show higher body masses than hamsters housed in reproductively inhibiting short-day photoperiods. Previous studies have manipulated reproductive state and patterns of endogenous melatonin (i.e., an indolemine hormone whose release acts as a physiological signal of photoperiod) to determine their contributions to seasonal variation in sickness intensity in Siberian hamsters (Freeman et al., 2007; Prendergast et al., 2008; Wen et al., 2007). While these manipulations modulated some sickness symptoms, they also resulted in body mass changes (Bilbo and Nelson, 2002; Prendergast et al., 2008; Wen et al., 2007). Therefore, while there were clearly effects of reproductive state and melatonin on seasonal variation in sickness, it is unclear if intensity was mediated directly by the manipulations or indirectly through changes in energetic state.

In a previous study, we manipulated a hormonal correlate of energetic state (i.e., leptin) in order to disentangle the effects of seasonal energetic changes from other seasonally modulated variables on sickness intensity variation (Carlton and Demas, 2014). Leptin levels are directly proportional with adipose tissue mass in mammals (Johnson et al., 2004; Maffei et al., 1995), and leptin changes seasonally, in parallel with seasonal changes in body mass, in Siberian hamsters and other seasonally breeding animals (Concannon et al., 2001; Gaspar-Lopez et al., 2009; Horton et al., 2000). We experimentally elevated circulating leptin levels in short-day Siberian hamsters so that they were comparable to long-day levels. We found that leptin treatment resulted in short-day hamsters showing hypothermic responses to LPS that were more long-day-like. Short-day hamsters treated with leptin,

however, showed LPS-induced anorexia that was not enhanced to the levels of long-day animals, but instead, was attenuated even below that of typical short-day levels. These results suggest that leptin modulates some but not all aspects of seasonal sickness variation. As leptin is only one of many energetic hormones, our findings did not eliminate the hypothesis that seasonal variation in sickness intensity is mediated by changes in energy stores. The goals of the present study were to manipulate seasonal energy stores, by using food restriction in long-day hamsters to mimic the pattern of seasonal body mass loss in short-day hamsters, to determine its effects on sickness intensity and to elucidate a potential hormonal mechanism mediating any changes in intensity. If seasonal variation in sickness intensity is driven by seasonal changes in energy stores, then we expected long-day hamsters displaying body mass loss patterns like short-day hamsters to show similar sickness intensity to short-day hamsters and less intensity than long-day hamsters fed *ad libitum*.

Methods

Animals and housing conditions

Adult male (> 60 days of age; average age = 156 days) Siberian hamsters (n = 90) were obtained from our breeding colony at Indiana University. All animals were initially group housed (2-5 per cage with same sex siblings on weaning at 17-18 days of age) in long-day photoperiods (light:dark (L:D) 16:8), and then individually housed in polypropylene cages (27.8 x 17.5 x 13.0 cm) for one week prior to experimental housing. Food (Laboratory Rodent Diet 5001, LabDiet, St.

Louis, MO, USA) and water were available *ad libitum* prior to the start of the experiment. Temperature (20 ± 2°C) and humidity (50 ± 10%) were maintained at constant levels. All animal methods were reviewed and approved by the Institutional Animal Care and Use Committee at Indiana University.

Experimental methods

Animals were assigned to one of three groups matched for initial body mass. The first group was housed in short days (L:D 8:16) and was fed *ad libitum* throughout the entire experiment (SD-AdLib; n = 46). Measures of body mass (to the nearest 0.1 g) and food consumption (to the nearest 0.1 g) were collected every other day for 10 weeks to track short-day induced changes in body mass and food intake and photoperiodic responsiveness to short days (described below). Food intake was assessed by weighing the food pellets remaining in the hopper each day.

The second and third groups were housed in long days (L:D 16:8) for the length of the experiment. The second group was fed *ad libitum* throughout the entire experiment (LD-AdLib; n = 20). Body mass and food intake were measured every other day for 10 weeks. The third group was provided *ad libitum* access to food for the first 10 days and then food restricted for the following 60 days (LD-FR; n = 24). During the first 10 days, body mass and food intake was measured every other day to establish pre-restriction mean values for these measures. During the next 60 days, these animals were allocated a pre-measured amount of food each day at the start of their active dark phase (1600 h) that ranged in quantities from 65-100% of the animal's pre-restriction mean food intake (Mauer and Bartness, 1997). Body

mass was recorded every other day for this group, and food access was adjusted in to keep the LD-FR group mean body mass tracking the SD-AdLib group mean. We modulated food availability to the LD-FR group to target the pattern of body mass loss in the SD-AdLib group, rather than providing food quantities that were pair matched to SD-AdLib food intake, because body mass loss in short days is both food intake dependent and independent (Wade and Bartness, 1984).

The SD-AdLib group was housed in experimental conditions six weeks prior to the LD-AdLib and LD-FR groups because a subset of hamsters housed in short days fails to show reproductive responsiveness to prolonged exposure to this photoperiod (i.e., do not display gonadal regression or reductions in body mass and fat stores). These individuals are referred to as photoperiodic non-responders (Puchalski and Lynch, 1986). Because we wanted to directly match body mass loss of the SD-AdLib and LD-FR groups, we needed to exclude the photoperiodic non-responders from our calculations of the SD-AdLib body mass loss trajectory (Mauer and Bartness, 1997). By observing body mass loss patterns in the short-day hamsters during the six weeks prior to housing the LD-FR group, we could remove animals who were not losing weight (and were likely photoperiodic non-responders) from the SD-AdLib body mass group means. Paired testes mass was collected at the end of the experiment in order to confirm short-day responsiveness (defined as a paired testes mass < 0.15 g) (Greives et al., 2008). Twelve animals were determined to be photoperiodic non-responders and removed from the experiment.

Starting on experimental day 70 for each group, body mass and food intake measurements were collected daily for the next 5 days to establish pre-injection baseline values for these measures. During these 5 days, at the start of the dark phase, LD-FR animals were provided daily food allocations equal to 100% of their pre-restriction means in order to relieve the effects of food restriction but not result in excessive food hoarding. After periods of food restriction, Siberian hamsters do not increase their food intake above normal levels when provided *ad libitum* access to food but do increase food hoarding (Bartness and Clein, 1994). In order to avoid complications with increased hoarding, we only provided hamsters access to their normal food intake rather than access to excess food that would be hoarded rather than consumed.

On the fifth day of baseline measurement collection, half of the animals in each group were injected intraperitoneally (i.p.) ~15 minutes before the onset of darkness with 25 µg LPS (LPS from *Salmonella enterica* serotype typhimurium, Sigma-Aldrich, St. Louis, MO, USA; Carlton and Demas, 2014) suspended in 0.1 ml sterile 0.9% saline. The remaining animals were injected i.p. with 0.1 ml sterile 0.9% saline. Sickness responses were assessed throughout the four days following injections.

Sickness response measurements

Fever, body mass loss, anorexia

Colonic temperatures (to the nearest 0.1°C) were collected immediately before injection and 2, 4, 6, 8, 10, 12, 16, 20, and 24 h after injection using a

MicroTherma 2T thermometer (ThermoWorks, Alpine, UT, USA) and a lubricated RET-3-ISO thermocouple probe (Physitemp Instruments, Inc., Clifton, NJ, USA) inserted ~12 mm into the rectum. To assess body mass loss and anorexia, daily body mass and food intake measurements continued until the end of the study. Hamsters in the SD-AdLib and LD-AdLib groups received *ad libitum* access to food until the end of the experiment, while hamsters in the LD-FR group continued to receive daily food allocations equal to 100% of their pre-restriction mean values.

Hedonic behavior

To assess the effects of our treatments on hedonic behavior, we provided hamsters with a highly palatable sodium saccharin solution (Baillie and Prendergast, 2008). Saccharin is a non-caloric artificial sweetener. As such, differences in ingestion between the groups would not interfere with our abilities to control energy intake. Beginning 5 days before injection, for the first 6 h of the dark phase (1600 h to 2200 h) hamsters were provided with a fluid bottle containing a solution of 0.1% sodium saccharin (saccharin sodium salt hydrate, Sigma-Aldrich, St. Louis, MO, USA) dissolved in tap water (Baillie and Prendergast, 2008). The saccharin solution bottles were weighed (to the nearest 0.1 g) before they were given and after they were collected from the hamsters each day. Presentation of saccharin solution continued daily through day 3 post-injection.

Nest building behavior

To assess the effects of our treatments on thermoregulatory behavior, beginning five days before injection, each hamster was provided with a compressed cotton nestlet weighing ~2.5 g (Ancare, Bellmore, NY, USA) for the first 6 h of the dark phase (Baillie and Prendergast, 2008). The nestlet was weighed (to the nearest 0.1 g) before presentation, and the unshredded portion was weighed after presentation. When provided a nestlet, hamsters quickly start shredding the cotton to build a nest. Nest building is an adaptive behavior to enhance energy conservation in low temperatures, however, hamsters readily build nests in room temperature (20-23°C) (Puchalski et al., 1988). Presentation of nestlets continued daily through day 3 post-injection.

Blood sampling and necropsies

Blood samples (~250 µl) were drawn from each animal 4 h after the onset of darkness at two time points (three days before injection and on the day of injection) to assess circulating blood glucose, leptin, and cortisol concentrations. Briefly, animals were lightly anesthetized with isoflurane vapors, and blood samples were drawn from the retro-orbital sinus. Blood samples were allowed to clot at room temperature for 1 h, clots were removed, and samples were centrifuged at 4°C for 30 min at 2500 rpm. Serum aliquots were aspirated and stored in sealable polypropylene microcentrifuge tubes at -20°C until assayed. All blood samples were collected within 3 min of initial handling. Animals were euthanized five days after LPS injection and necropsies were performed. Testes, inguinal white adipose

tissue (IWAT), epididymal WAT (EWAT), and retroperitoneal WAT (RWAT) were removed, cleaned of connective tissues, and weighed to the nearest 0.1 mg. A composite adipose tissue score was calculated by summing the individual WAT pad masses.

Blood glucose measurement

Blood glucose was measured from the samples collected 4 hours after injection. Immediately upon collection, ~5 µl of whole blood was transferred onto test strips of a blood glucose monitoring system (ReliOn, Micro Blood Glucose Monitoring System, Arkray USA, Inc., Minneapolis, MN, USA), and the readout was recorded. The meter was previously calibrated using an internal standard provided by the manufacturer.

Leptin enzyme-linked immunosorbent assay (ELISA)

We assessed circulating leptin levels in the samples collected three days prior to injection to determine if the groups showed differing serum concentrations of this hormone. Leptin levels were assayed via commercially prepared mouse leptin ELISA kits (Crystal Chem, Downers Grove, IL, USA). This kit has previously been validated in Siberian hamsters (Carlton and Demas, 2014). Samples were diluted 1:4 and run in duplicate. Intra-assay variabilities were 6.8%, 12.5%, and 1.8%.

Cortisol enzyme immunoassay (EIA)

We assessed circulating cortisol levels to determine if the groups differed in the magnitude of baseline and LPS-induced activation of the HPA axis. Cortisol is the predominant glucocorticoid in Siberian hamsters (Reburn and Wynne-Edwards, 2000). Serum cortisol concentrations were determined in multiple EIAs from a commercially prepared kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA). This assay was previously validated for use in Siberian hamsters (Demas et al., 2004). Samples were diluted to 1:80 with assay buffer and run in duplicate. Intra-assay variabilities were 3.1%, 0%, and 0.6%.

Statistical analyses

All statistics were performed using JMP 10 (SAS Institute Inc., Cary, NC, USA). Residuals were checked for normality and homogeneity of variance, and those data that were non-normally distributed were transformed with the function that best fit the data. Three animals were excluded from the final analyses: one from the saline-treated SD-AdLib group because it exhibited sickness symptoms despite receiving no LPS, one from the LPS-treated LD-AdLib group because it showed abnormal body mass loss despite no food restriction, and one from the saline-treated LD-AdLib group because it displayed abnormal food hoarding. The final sample sizes were as follows: SD-AdLib-Saline (n = 16), SD-AdLib-LPS (n=17), LD-AdLib-Saline (n=9), LD-AdLib-LPS (n=9), LD-FR-Saline (n=12), LD-FR-LPS (n=12).

Pre-injection baseline values were calculated for body mass, food intake, saccharin solution intake, and percent nesting material shredded by averaging the three daily measurements immediately prior to injections (days 72-74). We did not include days 70-71 in this mean because measures on these days were more variable as animals were adjusting to changes in food allocations and the presence of saccharin solution and nestlets. To determine if there were group effects on pre-injection baseline body mass, baseline food intake, leptin levels, and baseline saccharin solution intake, one-way analyses of variance (ANOVA) were performed. Pre-injection baseline saccharin solution intake was log transformed and leptin concentration was square root transformed. Pre-injection baseline percent nesting material shredded could not be transformed to meet the assumptions of normality, so a Kruskal-Wallis test was performed.

Because group affected pre-injection food intake, body mass, saccharin solution intake, and percent nesting material shredded (see Results), post-injection changes in these measurements were expressed as percentages of each animal's baseline values. Repeated measures ANOVAs were performed on post-injection percent changes in food intake, body mass, and saccharin solution intake and colonic temperature. The within-subject comparisons for percent change in body mass, colonic temperature, and percent change in saccharin solution intake violated the assumptions of sphericity and were Greenhouse-Geisser corrected. Post-injection percent change in saccharin solution intake was square root transformed. Post-injection changes in percent nesting material shredded could not be transformed to meet the assumptions of normality, so a Kruskal-Wallis test was

performed. Differences in glucose, cortisol, and tissue masses among the groups were assessed with two-way ANOVAs. Glucose was square root transformed, while cortisol and the tissue masses were log transformed. Correlations between pre-injection baseline body mass and percent changes in body mass were assessed for LPS-treated animals using analyses of covariance (ANCOVA). Post-hoc comparisons were conducted using Fisher's Least Significant Difference tests when ANOVAs were statistically significant.

Results

An experimental timeline is presented in Figure 1 in order to clarify at which timepoints measures were collected.

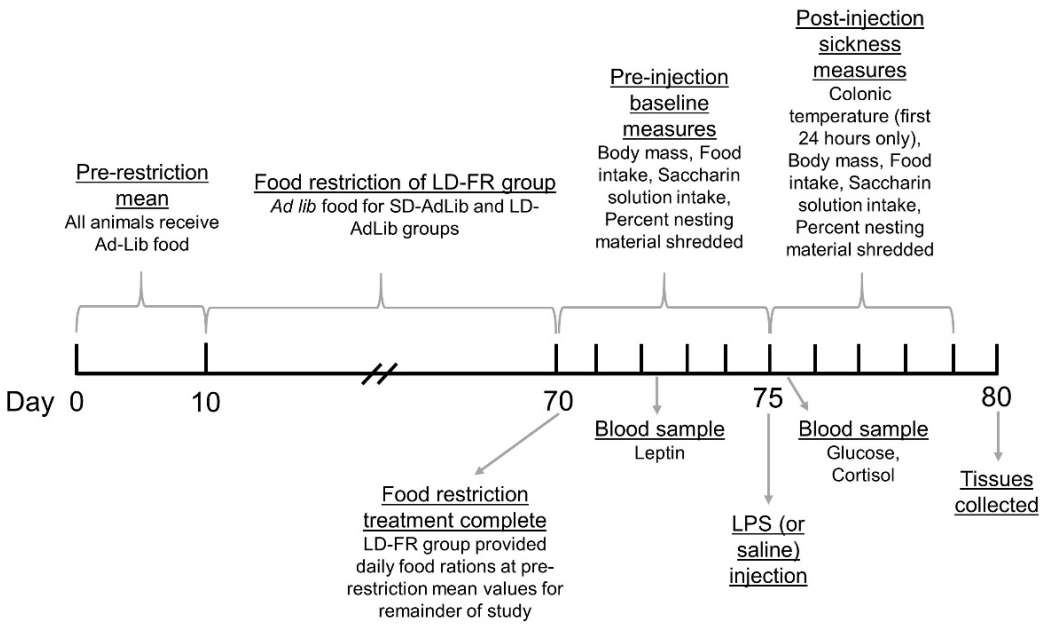

Figure 1. Experimental timeline indicating when treatments were performed and when measures were collected. Day 0 refers to the timepoint when each group was housed in their experimental photoperiods, after one week of acclimation to individual housing.

Experimental induction of body mass loss

Body mass did not differ among the groups prior to experimental housing ($F_{2,72}$ = 0.281, P = 0.756). After 70 days in experimental photoperiods and food restriction for the long-day food-restricted group (LD-FR), body mass differed among the groups ($F_{2,72}$ = 25.372, P < 0.001) (Fig. 2). Long-day *ad libitum* (LD-AdLib) hamsters showed no change in body mass from days 0 to 70 (Paired T = 0.230, P = 0.821), while short-day *ad libitum* (SD-AdLib) and LD-FR hamsters showed 22.3% and 22.0% decreases in body mass, respectively. There were no differences in body mass at day 70 between the LD-FR and SD-AdLib groups (T_{17} = 0.117, P = 0.907).

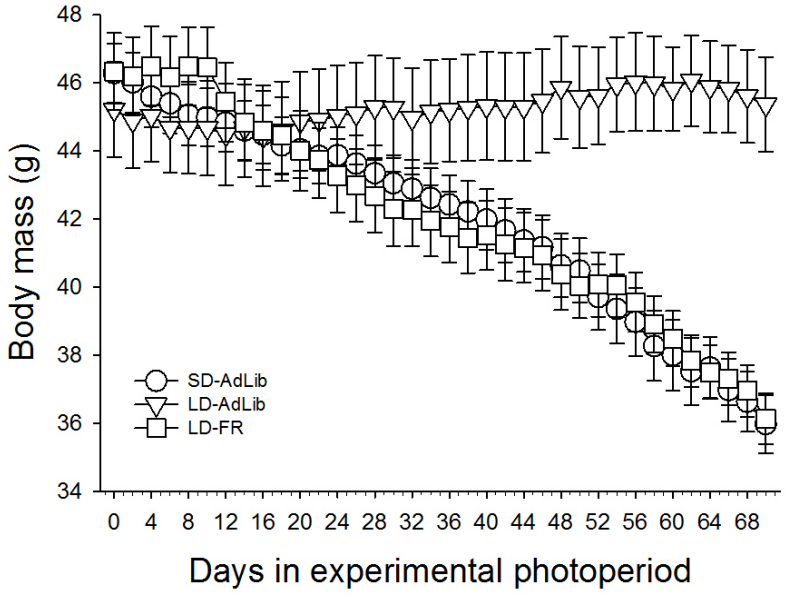

Figure 2. Mean (± SEM) body mass of male Siberian hamsters over the course of the first 70 days of experimental housing. Hamsters were either allowed *ad libitum* access to food during the experiment and housed in short- (SD-AdLib) or long-day (LD-AdLib) photoperiods or provided restricted access to food and housed in long-day photoperiod (LD-FR).

Pre-injection baseline measures

At the end of those 70 days and until the end of the experiment, LD-FR animals were allocated food at their pre-restriction mean values. Pre-injection baseline food consumption differed among the groups ($F_{2,72}$ = 12.143, P < 0.001) (Table 1). Specifically, SD-AdLib hamsters consumed less food than LD-AdLib and LD-FR hamsters (T > 3.560, P < 0.001 for both comparisons), while there was no difference in consumption between the LD-AdLib and LD-FR hamsters (T = 1.181, P = 0.241). LD-FR body mass did increase slightly upon access to pre-restriction mean food levels (Paired T = 5.105, P < 0.001); however, pre-injection baseline body masses still did not differ between the LD-FR and SD-AdLib groups (T = 1.495, P = 0.139) (Table 1). While body mass did not differ between the LD-FR and SD-AdLib groups, serum leptin levels differed among all three groups ($F_{2,72}$ = 25.412, P < 0.001) (Table 1). Specifically, LD-AdLib hamsters showed the highest leptin concentrations, while LD-FR and SD-AdLib hamsters had leptin concentrations 33.5% and 67.1%, respectively, lower than this group.

Pre-injection baseline saccharin solution intake varied among the groups ($F_{2,72}$ = 16.917, P < 0.001) (Table 1). Specifically, LD-FR animals consumed greater volumes of saccharin solution as compared to LD-AdLib (T = 3.428, P = 0.001) and SD-AdLib (T = 5.782, P < 0.001) animals. There was no difference in pre-injection baseline saccharin solution intake between the LD-AdLib and SD-AdLib groups (T = 1.646, P = 0.104). Pre-injection baseline percent nesting material shredded also differed by group prior to injection (H = 15.105, P < 0.001) (Table 1). Specifically, LD-FR animals shredded a lower percentage of their cotton

nestlet as compared to LD-AdLib ($Z = 1.987$, $P = 0.047$) and SD-AdLib ($Z = 3.813$, $P < 0.001$) animals. There was no difference in percent nesting material shredded between the LD-FR and SD-AdLib groups ($Z = 1.136$, $P = 0.256$).

Table 1. Mean (± SEM) pre-injection baseline food intake, baseline body mass, circulating serum leptin concentrations, baseline saccharin solution intake, and baseline percent nesting material shredded in short-day *ad libitum* fed (SD-AdLib), long-day *ad libitum* fed (LD-AdLib), and long-day (previously) food restricted (LD-FR) male Siberian hamsters. Pre-injection baseline measurements were calculated by averaging the values collected for the measurements during the three days prior to LPS or saline injection. During the five days prior to injection, animals in the LD-FR group were allocated food at 100% of their pre-restriction mean values. Groups with different letters indicate statistically significant differences between group means ($P < 0.05$); groups sharing the same letter are statistically equivalent.

	SD-AdLib	**LD-AdLib**	**LD-FR**
Baseline Food Intake (g/day)	4.1 ± 0.1^{a}	5.0 ± 0.2^{b}	4.8 ± 0.1^{b}
Baseline Body Mass (g)	35.5 ± 0.8^{a}	45.3 ± 1.4^{b}	37.3 ± 0.7^{a}
Leptin conc. (pg/ml)	7.07 ± 0.96^{a}	18.88 ± 1.41^{c}	12.90 ± 1.15^{b}
Baseline Saccharin Solution Intake (g / 6 h)	1.5 ± 0.1^{a}	1.8 ± 0.2^{a}	3.0 ± 0.3^{b}
Baseline Percent Nesting Material Shredded	99.0 ± 0.5^{a}	93.8 ± 3.2^{a}	85.9 ± 4.1^{b}

Post-injection sickness measures

Anorexia

Percent changes in food intake were affected by group ($F_{2,69}$ = 5.325, P = 0.007) and by injection ($F_{1,69}$ = 158.030, P < 0.001) but not by the group x injection interaction ($F_{2,69}$ = 0.314, P = 0.731) (Fig. 3). Percent changes in food intake varied across the four days after injection (within subjects, $F_{3,67}$ = 157.130, P < 0.001) and with the time x group ($F_{6,134}$ = 3.521, P = 0.003) and the time x injection ($F_{3,67}$ = 157.130, P < 0.001) interactions. All LPS-treated animals showed greater decreases in food intake compared to their respective saline-treated controls at days 1, 2, and 3 post-injection (P < 0.003 for all comparisons). By day 4, the LPS-treated LD-FR and SD-AdLib groups no longer showed reduced food intake compared to controls (P > 0.06 for both comparisons); however, the LPS-treated LD-AdLib group still showed reduced food intake on this day as compared to controls (P = 0.006). Additionally, at day 4 post-injection, the LPS-treated LD-AdLib group showed greater percent decreases in food intake than the LPS-treated SD-AdLib group (P = 0.016) but not the LD-FR group (P = 0.149).

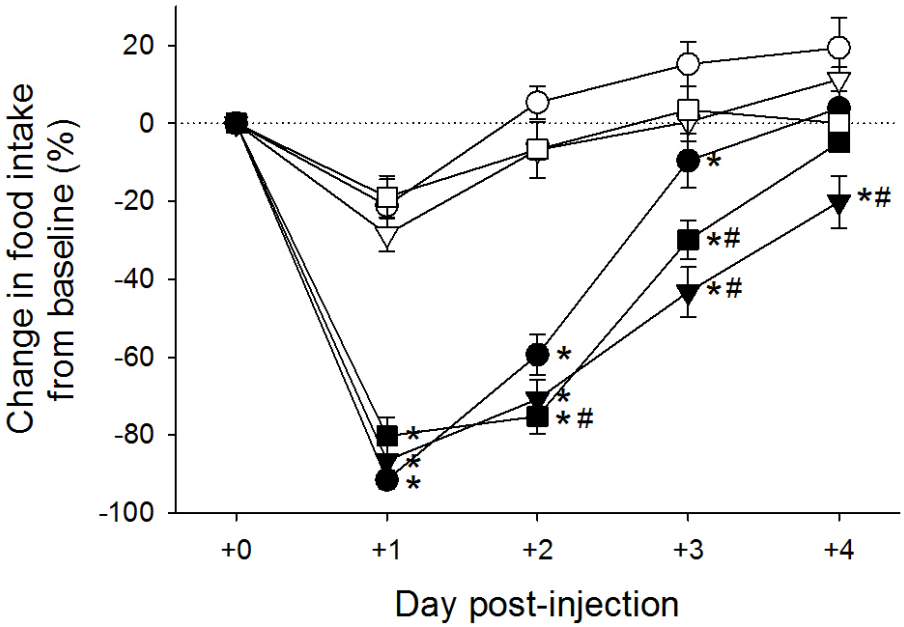

Figure 3. Mean (± SEM) percent change in daily food intake from baseline following LPS (black icons) or saline (white icons) treatment delivered on day 0 in short-day *ad libitum* fed (SD-AdLib,), long-day *ad libitum* fed (LD-AdLib,), and long-day (previously) food restricted (LD-FR,) male Siberian hamsters. Day +1 represents the time period from 0-24 h after LPS or saline injection, Day +2 represents the time period from 24-48 h after LPS or saline injection, and so forth. $^{*}P < 0.05$ versus saline-treated group exposed to the same photoperiod and food treatments. $^{\#}P < 0.05$ versus SD-AdLib LPS-treated group.

Body mass loss

Percent changes in body mass were affected by group ($F_{2,69} = 11.896$, $P < 0.001$) and by injection ($F_{1,69} = 174.759$, $P < 0.001$) but not by the group x injection interaction ($F_{2,69} = 1.034$, $P = 0.361$) (Fig. 4). Percent decreases in body mass changed across the four days post injection (within subjects, $F_{2.2,149.4} = 7.545$, $P < 0.001$) and with the time x group ($F_{4.3,149.4} = 2.809$, $P = 0.024$), time x injection ($F_{2.2,149.4} = 12.564$, $P < 0.001$), and time x group x injection ($F_{4.3,149.4} = 4.096$, $P = 0.003$) interactions. At all 4 days after injection, LPS-treated animals showed greater percent decreases in body mass as compared to their respective saline-

treated controls ($P < 0.001$ for all comparisons); however, the LPS-treated LD-FR and SD-AdLib groups showed attenuated percent decreases in body mass relative to the LPS-treated LD-AdLib group. Specifically, the LD-FR group showed lesser percent body mass decreases as compared to the LD-AdLib group on days 2, 3, and 4 post-LPS injection ($P < 0.02$ for all comparisons), while the SD-AdLib group showed lesser decreases in percent body mass loss on days 3 and 4 post-LPS injection ($P < 0.002$ for both comparisons).

In LPS-treated animals, percent change in body mass was negatively correlated with pre-injection baseline body mass, such that animals with greater initial body masses showed greater percent decreases in body mass on days 3 (body mass: $F_{1, 34} = 9.011$, $P = 0.005$; group: $F_{2, 34} = 2.644$, $P = 0.086$) and 4 (body mass: $F_{1, 34} = 6.037$, $P = 0.019$; group: $F_{2, 34} = 3.771$, $P = 0.033$) but not days 1 or 2 ($P > 0.05$ for body mass on both days). In addition, the maximum percent change in body mass that each LPS-treated animal displayed after injection was not correlated with pre-injection baseline body mass (body mass: $F_{1, 34} = 1.890$, $P = 0.178$; group: $F_{2, 34} = 2.852$, $P = 0.0716$).

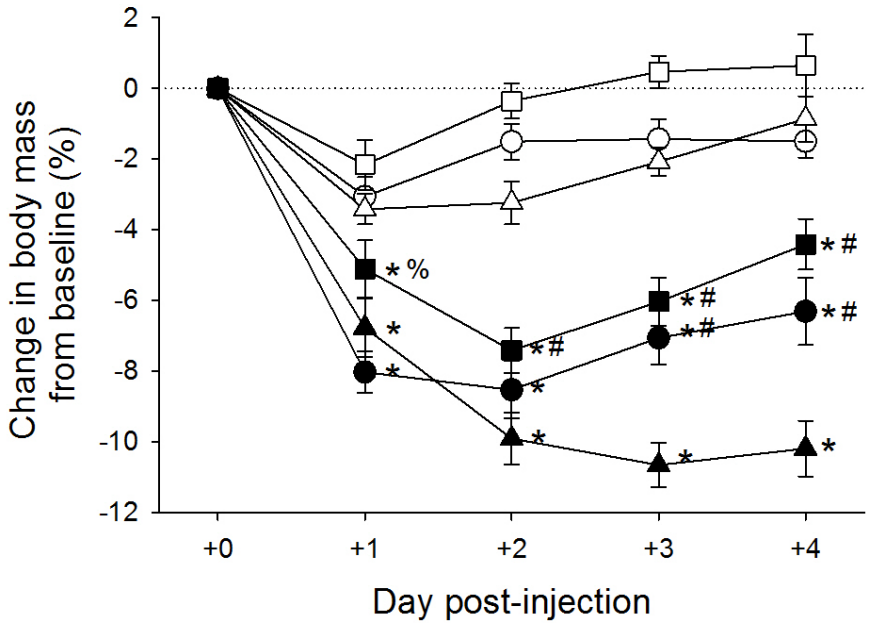

Figure 4. Mean (± SEM) percent change in body mass from baseline following LPS (black icons) or saline (white icons) treatment delivered on day 0 in short-day *ad libitum* fed (SD-AdLib,), long-day *ad libitum* fed (LD-AdLib,), and long-day (previously) food restricted (LD-FR,) male Siberian hamsters. Day +1 represents the time period from 0-24 h after LPS or saline injection, Day +2 represents the time period from 24-48 h after LPS or saline injection, and so forth. $^{*}P < 0.05$ versus saline-treated group exposed to the same photoperiod and food treatments. $^{\#}P < 0.05$ versus LD-AdLib LPS-treated group. $^{\%}P < 0.05$ versus SD-AdLib LPS-treated group.

Body temperature

Colonic temperature differed by group ($F_{2,69}$ = 7.368, P = 0.001) and injection ($F_{1,69}$ = 44.515, P < 0.001) but not by the group x injection interaction ($F_{2,69}$ = 1.302, P = 0.279) (Fig. 5). Colonic temperature changed across time (within subjects, $F_{6.0,415.4}$ = 180.880, P < 0.001) and with the time x injection ($F_{6.0,415.4}$ = 34.795, P < 0.001) and time x group x injection ($F_{2.3,12,0}$ = 2.329, P = 0.007) interactions. Among saline-injected animals, the SD-AdLib group showed higher

temperatures than the LD-AdLib and LD-FR groups at 8, 10, and 16 h post-injection ($P < 0.05$). LPS injection resulted in a hypothermic response across all groups at most time points after injection. However, at 2 h post-injection, LPS-treated SD-AdLib and LD-FR hamsters showed greater temperatures as compared to their respective saline controls ($P < 0.003$); the temperature of LPS-treated LD-AdLib hamsters did not differ from that of the saline-treated controls at this time point ($P = 0.369$). LPS-treated animals in all groups exhibited hypothermic responses as compared to their respective saline controls starting at either 4 h post-injection (LD-AdLib) or 6 h post-injection (SD-AdLib and LD-FR) throughout the end of the 24 h measurement period ($P < 0.05$ for all comparisons except SD-AdLib at 20 h post-injection). The temperature of the LPS-treated LD-AdLib group was lower than the temperature of the LPS-treated SD-AdLib group at 4, 8, 10, and 16 h post-injection *(*$P < 0.05$ for these comparisons*)*, while the temperature of the LD-FR group was only lower than that of the SD-AdLib group at 8 h post-injection ($P = 0.016$).

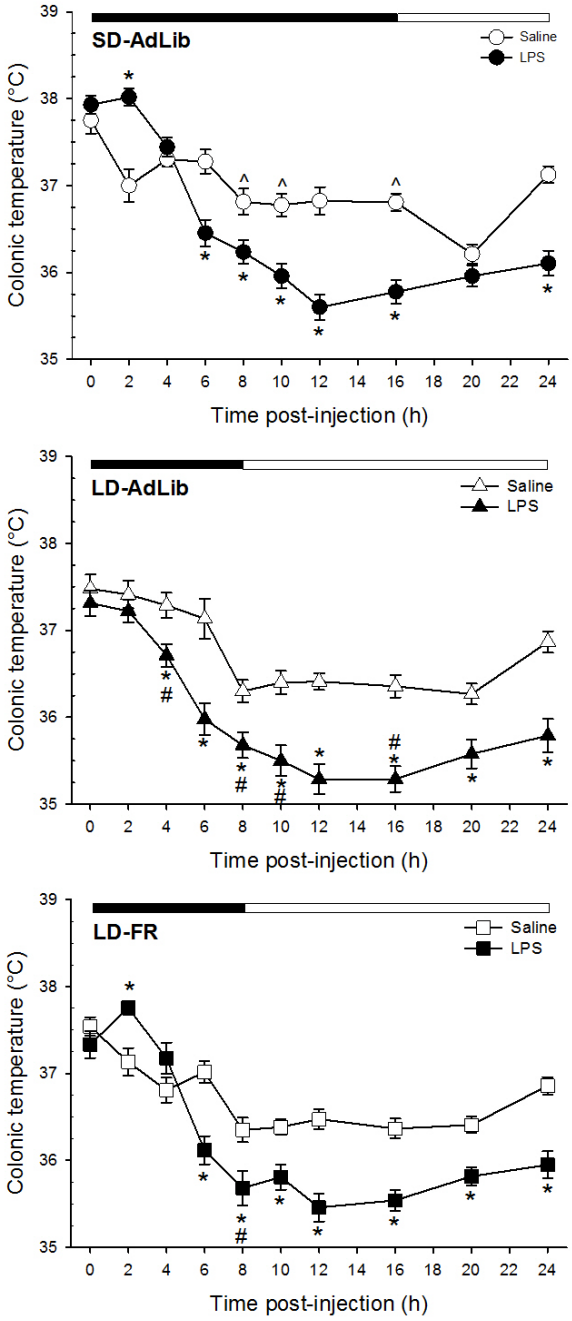

Figure 5. Mean (± SEM) colonic temperature following LPS and saline treatments delivered at the 0 h time point in short-day *ad libitum* fed (SD-AdLib, top panel), long-day *ad libitum* fed (LD-AdLib, middle panel), and long-day (previously) food restricted (LD-FR, bottom panel) male Siberian hamsters. Black and white bars at the top of the graphs indicate the active, dark (black) and inactive, light (white) phases of the light-dark cycle for each photoperiodic morph. Within each panel: $^{*}P < 0.05$ versus saline-treated group exposed to the same photoperiod and food treatments. Across panels: $^{\#}P < 0.05$ versus LPS-treated SD-AdLib group. $^{\wedge}P < 0.05$ from saline-treated LD-AdLib and LD-FR groups.

Saccharin solution intake

Percent change in saccharin solution intake was affected by injection treatment only ($F_{1,69}$ = 7.600, P = 0.008) and not by group ($F_{2,69}$ = 1.089, P = 0.342) or the group x injection interaction ($F_{2,69}$ = 0.885, P = 0.418) (Fig. 6A). Percent change in saccharin solution intake varied across time (within subjects, $F_{2.5,173.8}$ = 3.674, P = 0.019) and with the time x group ($F_{5.0,173.8}$ = 4.966, P < 0.001) and time x injection ($F_{2.5,173.8}$ = 9.081, P < 0.001) interactions. While there was considerable variance in this measure, post hoc analyses revealed that saccharin solution intake was reduced in the LPS-treated LD-AdLib and LD-FR groups during the 0-6 h timepoint as compared to their respective saline-treated controls (P < 0.009 for both comparisons).

Nest building behavior

Percent change in nesting material shredded differed among the groups from the 0-6 h (H = 43.543, P < 0.001), 24-30 h (H = 59.684, P < 0.001), and 48-54 h (H = 50.964, P < 0.001) timepoints but not at the 72-78 h timepoint (H = 4.356, P = 0.499) (Fig. 6B). Specifically, at the 0-6 h, 24-30 h, and 48-54 h timepoints, all three LPS-treated groups showed decreases in nesting material shredded as compared to their respective saline-treated controls (Z > 3.003, P < 0.002 for all comparisons); however, LPS-treated LD-FR animals showed lesser percent decreases in nesting material shredded than the LPS-treated SD-AdLib group at the 24-30 h (Z = 2.483, P = 0.013) and 48-54 h (Z = 2.037, P = 0.042) timepoints and the LPS-treated LD-AdLib group at the 24-30 h timepoint (Z = 2.111, P = 0.035).

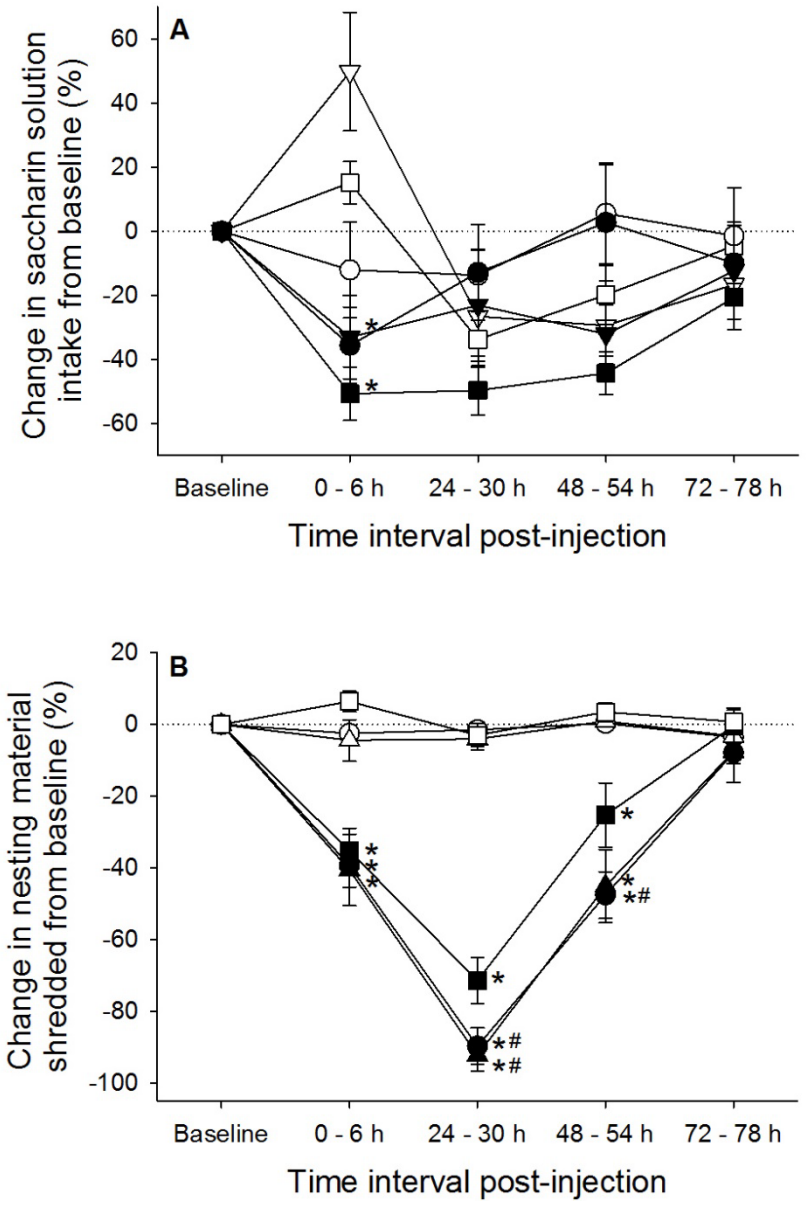

Figure 6. Mean (± SEM) percent changes in (A) saccharin solution intake and (B) nesting material shredded from baseline following LPS (black icons) and saline treatment (white icons) delivered at the 0 h time point in short-day *ad libitum* fed (SD-AdLib, ●-), long-day *ad libitum* fed (LD-AdLib, ▲-), and long-day (previously) food restricted (LD-FR, ■-) male Siberian hamsters. $^{*}P < 0.05$ versus saline-treated group exposed to the same photoperiod and food treatments. $^{\#}P < 0.05$ versus LD-FR LPS-treated group.

Blood glucose

Blood glucose levels were affected by group ($F_{2,69}$ = 3.77, P = 0.028) and injection ($F_{1,69}$ = 114.23, P < 0.001) but not the group x injection interaction ($F_{2,69}$ = 0.24, P = 0.784) (Fig. 7A). All LPS-treated hamsters showed lower blood glucose levels than their respective saline-treated controls (T > 5.75, P < 0.001 for all comparisons). Additionally, saline-treated LD-FR hamsters had lower blood glucose concentrations than saline-treated LD-AdLib hamsters (T = 2.42, P = 0.018).

Cortisol

Cortisol was affected by group ($F_{2,69}$ = 18.082, P < 0.001) and injection ($F_{1,69}$ = 65.005, P < 0.001) but not the group x injection interaction ($F_{2,69}$ = 0.402, P = 0.671) (Fig. 7B). LPS-treated animals from all groups showed elevated cortisol levels compared to their respective saline-treated controls (P < 0.001 for all comparisons). LPS-treated LD-FR and SD-AdLib animals had higher cortisol levels than LPS-treated LD-AdLib animals (P < 0.02 for both comparisons), while saline-treated LD-FR and SD-AdLib animals had higher cortisol levels than saline-treated LD-AdLib animals (P < 0.006 for both comparisons). Cortisol levels of the LPS-treated LD-AdLib animals were similar to those of saline-treated LD-FR and SD-AdLib animals (P > 0.05 for both comparisons).

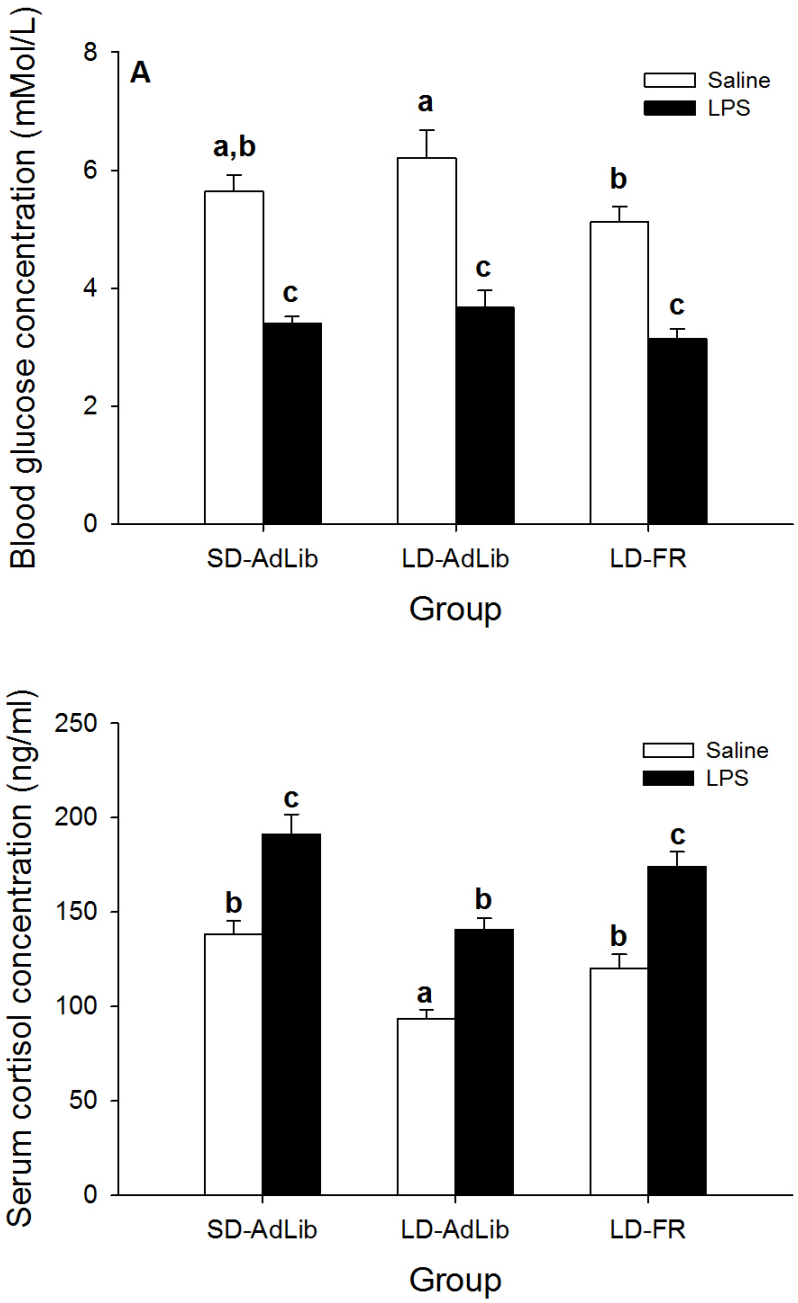

Figure 7. Mean (± SEM) (A) blood glucose and (B) circulating serum cortisol concentrations taken 4 h following LPS and saline treatments in short-day *ad libitum* fed (SD-AdLib), long-day *ad libitum* fed (LD-AdLib), and long-day (previously) food restricted (LD-FR) male Siberian hamsters. Within each panel, groups with different letters indicate statistically significant differences between group means ($P < 0.05$); groups sharing the same letter are statistically equivalent.

Tissue masses

Paired testes mass was affected by group ($F_{2,69}$ = 1425.837, P < 0.001) but not by injection ($F_{1,69}$ = 2.454, P = 0.122) or the group x injection interaction ($F_{2,69}$ = 0.373, P = 0.690) (Table 2). Specifically, SD-AdLib hamsters had paired testes masses that were regressed in comparison to LD-AdLib and LD-FR hamsters regardless of injection treatment (P < 0.001 for all comparisons). IWAT, EWAT, RWAT, and composite fat masses were affected by group ($F_{2,69}$ > 15.69, P < 0.001 for all models), but not injection ($F_{1,69}$ < 0.07, P > 0.806 for all models) or the group x injection interaction ($F_{2,69}$ < 0.23, P > 0.799 for all models) (Table 2). All groups showed significantly different composite fat masses from each other, with the LD-AdLib groups having the greatest composite fat mass, followed by the LD-FR groups, and then the SD-AdLib groups (P < 0.02 for all comparisons between groups).

Table 2. Mean (± SEM) paired testes, inguinal white adipose tissue (IWAT), epididymal WAT (EWAT), retroperitoneal WAT (RWAT), and composite adipose tissue masses from short-day *ad libitum* fed (SD-AdLib), long-day *ad libitum* fed (LD-AdLib), and long-day (previously) food restricted (LD-FR) male Siberian hamsters. Tissue masses were collected via necropsy at the conclusion of the experiment (five days after LPS or saline injection). Groups with different letters indicate statistically significant differences between group means ($P < 0.05$); groups sharing the same letter are statistically equivalent.

	SD-AdLib	LD-AdLib	LD-FR
Paired testes mass (g)			
Saline	0.049 ± 0.004^{a}	0.778 ± 0.042^{c}	0.623 ± 0.025^{b}
LPS	0.046 ± 0.003^{a}	$0.671 \pm 0.033^{b,c}$	0.606 ± 0.028^{b}
IWAT mass (g)			
Saline	0.488 ± 0.084^{a}	1.062 ± 0.141^{b}	0.627 ± 0.085^{a}
LPS	0.465 ± 0.077^{a}	1.083 ± 0.107^{b}	0.513 ± 0.054^{a}
EWAT mass (g)			
Saline	0.357 ± 0.044^{a}	$0.935 \pm 0.109^{b,c}$	0.624 ± 0.067^{b}
LPS	0.340 ± 0.049^{a}	0.999 ± 0.119^{c}	0.610 ± 0.055^{b}
RWAT mass (g)			
Saline	0.057 ± 0.009^{a}	0.164 ± 0.024^{e}	$0.106 \pm 0.019^{c,d}$
LPS	$0.064 \pm 0.011^{a,b}$	$0.155 \pm 0.018^{d,e}$	$0.087 \pm 0.012^{b,c}$
Composite adipose tissue mass (g)			
Saline	0.902 ± 0.133^{a}	2.160 ± 0.264^{c}	1.357 ± 0.166^{b}
LPS	0.869 ± 0.134^{a}	2.237 ± 0.237^{c}	1.210 ± 0.112^{b}

Discussion

The results of this study demonstrate that seasonal differences in body mass alone do not regulate all variation in sickness symptoms in Siberian hamsters; however, inducing body mass loss in long-day housed hamsters does result in animals displaying some symptoms that appear more short-day-like. Specifically, long-day hamsters that were food restricted showed body mass loss in response to

LPS that was attenuated in comparison to long-day *ad libitum* hamsters but comparable to short-day *ad libitum* hamsters. The attenuation of body mass loss in long-day food restricted hamsters was likely due in part to attenuation of LPS-induced anorexia, as the long-day restricted hamsters showed patterns of anorexia intermediate between the short-day and long-day *ad libitum* groups but showed the lowest percentage of body mass loss of the three groups. We expected to see differences in the magnitude of LPS-induced decreases in nest building between the short- and long-day *ad libitum* groups; however, no differences were observed. Rather, we found that the long-day restricted group showed less of a decrease in nest building after LPS, suggesting that in this experimental context, the act of prior food restriction may have had greater impacts than photoperiod on this measure. In contrast to our predictions, the intensity of LPS-induced anhedonic behavior was not affected by food restriction, as both long-day *ad libitum* and restricted animals showed LPS-induced decreases in saccharin solution intake at 0-6 h post-injection while short-day *ad libitum* animals did not.

All three LPS-treated groups showed hypothermia, rather than fever, from 4 or 6 hours post-injection until the end of the measuring period 24 hours after injection. Hypothermic responses to sickness are not uncommon (Burness et al., 2010; Carlton and Demas, 2014; French et al., 2013b; Martin et al., 2008b; Owen-Ashley et al., 2008) and can actually be beneficial to survival during severe inflammation or low energy availability (Romanovsky and Szekely, 1998). For instance, rats that receive high concentrations of endotoxin display hypothermic responses and also show decreased glucose levels compared to control-injected

animals; however, rats that receive lower doses of endotoxin show fever and no hypoglycemia (Lang et al., 1985). As all three LPS-treated groups showed reduced glucose in comparison to saline-treated animals after injection, their glucose levels coupled with their hypothermic responses may suggest the animals were experiencing severe inflammation. In concordance with the photoperiodic influence on temperature, hamsters in the LPS-treated long-day *ad libitum* group had colonic temperatures that were lower than the short-day group at several timepoints, indicating that photoperiodic influences on temperature were maintained during the hypothermic response. Alternatively, the LPS-treated long-day restricted group only had temperature recordings that were lower than the LPS-treated short-day group at 8 hours post-injection, suggesting that hypothermia was attenuated in this group as compared to the LPS-treated long-day *ad libitum* group.

We assessed correlations between pre-LPS baseline body mass and the maximum percent body mass loss that was displayed after injection. We did not find a significant correlation between these measures, suggesting that intensity of energetically costly sickness symptoms may not be entirely limited by a minimum body mass threshold that the animal cannot surpass in order to survive (Ashley and Wingfield, 2012). However, there were significant correlations between pre-LPS body mass and percent body mass loss at days 3 and 4 post-LPS. Whereas maximum percent body mass loss may not be correlated with pre-LPS body mass, correlations at days 3 and 4 post-LPS suggest that the length of time an animal can maintain body mass loss throughout sickness may be constrained by their pre-sickness body mass. In white-crowned sparrows (*Zonatrichia leucophrys*), baseline

body mass is correlated with post-LPS percent decreases in body mass, with initially heavier individuals showing the greatest decreases in body mass 24 hours after LPS injection (Owen-Ashley et al., 2008; Owen-Ashley et al., 2006). The differences in timepoints at which these correlations are observed between hamsters and sparrows may reflect species differences in the amount of extra energy reserves each animal stores. For instance, if hamsters maintain greater surplus energy stores than sparrows, then the need to regulate sickness symptoms to avoid hitting a body mass where survival is risked may not occur until later into the sickness response. In the future, doing comparative studies across species with varying degrees of surplus energy stores may elucidate relationships between body mass and sickness intensity.

Because we found that long-day restricted hamsters showed attenuation of some sickness symptoms, we sought to determine what energetic hormones could be acting as intermediaries between body mass and sickness. We previously showed that seasonal variation in leptin mediates seasonal variation in LPS-induced hypothermia but not other sickness symptoms (Carlton and Demas, 2014). In the present study, we found that even though the long-day restricted group showed comparable body masses to the short-day *ad libitum* group, long-day restricted animals had higher leptin levels than short-day *ad libitum* animals. Therefore, if leptin is the primary intermediary between body mass and sickness, then we would have expected to see that sickness symptoms exhibited by the long-day restricted group were in-between the short- and long-day *ad libitum* groups. The results of this present study are largely consistent with previous findings (Carlton and Demas,

2014), as the long-day restricted group showed an intermediate hypothermic response, exhibiting temperatures that fell inbetween those of the short-day and long-day *ad libitum* groups at several timepoints. The long-day restricted group also showed an intermediate anorexic response. While the intensity of this measure is consistent with differences in leptin levels across groups, our previous work directly manipulating leptin levels does not support the hypothesis that leptin mediates sickness-induced anorexia in Siberian hamsters (Carlton and Demas, 2014). Although some research has linked leptin with sickness-induced anorexia (Harden et al., 2006; Sachot et al., 2004), other studies have suggested that these variables have little relationship with each other (Faggioni et al., 1997; Lugarini et al., 2005). Thus, the role of leptin in modulating sickness-induced anorexia in Siberian hamsters and other species remains unresolved.

Suppression of the inflammatory response via circulating cortisol may be a promising mechanism to explain the attenuated sickness in the long-day restricted group. Glucocorticoids are released during sickness responses and are critical for regulating their intensity (Sapolsky et al., 2000). Rats that have their adrenal glands removed to prevent glucocorticoid production show greater body mass loss in response to LPS as compared to sham-operated controls (Johnson et al., 1996), while administration of synthetic glucocorticoids concurrently with LPS attenuates sickness-induced decreases in food intake (Uehara et al., 1989). Both the saline- and LPS-treated long-day restricted groups in our study exhibited elevated cortisol levels relative to their respective long-day *ad libitum* counterparts. Thus, it is possible that increased circulating cortisol levels in the long-day restricted animals

may have acted to suppress immunological modulators of sickness-induced anorexia and body mass loss.

Although the long-day restricted group exhibited elevated cortisol levels relative to the long-day *ad libitum* group, both the saline-treated and LPS-treated long-day restricted groups had similar cortisol levels to their respective short-day *ad libitum* counterparts. Siberian hamsters show increased circulating cortisol levels in short days as compared to long days (Ashley et al., 2013; Bilbo and Nelson, 2003; Carlton and Demas, 2014). Therefore, it is possible that increased cortisol levels may be facilitating the attenuation of sickness symptoms in short-day animals. Previous studies have investigated the effects of melatonin, testosterone, and leptin on seasonal variation in sickness intensity, but none have identified a seasonally modulated hormone that explains the majority of sickness response variation in this species (Bilbo and Nelson, 2002; Carlton and Demas, 2014; Prendergast et al., 2008; Wen et al., 2007). In addition, there may also be seasonal changes in glucocorticoid receptors or binding proteins in this species that may affect regulation of sickness responses. Although no studies have explored seasonal variation in glucocorticoid receptors and proteins in Siberian hamsters, there is evidence that they vary seasonally in other species and that seasonal variation in receptors can occur within immune tissues (Lattin et al., 2013; Romero et al., 2006) Thus, examining the roles that seasonal variation in glucocorticoid mechanism play in this phenomenon may be a promising next step.

While short-day hamsters displayed increased cortisol levels with *ad libitum* food access, we had to food restrict long-day hamsters to achieve the same

levels. Sustained moderate food restriction has been shown to increase cortisol levels in Siberian hamsters (Bilbo and Nelson, 2004; Zysling et al., 2009). Corticosterone is elevated to higher levels after LPS injection in food restricted mice and rats than in non-food restricted animals, and its elevation corresponds with lower levels of proinflammatory cytokines (MacDonald et al., 2014; Matsuzaki et al., 2001). Thus, it is possible that increased cortisol levels generated by food restriction may be suppressing proinflammatory cytokines in long-day restricted hamsters in this study.

It cannot be ignored that although we induced body mass loss in the long-day restricted group so that it mimicked natural short-day body mass loss, this pattern of body mass loss in long-day animals would likely be interpreted by the brain and periphery as "seasonally inappropriate" (Mercer et al., 2001). Differences in neural interpretation of energetic state between the long-day restricted and short-day *ad libitum* groups may explain why the long-day restricted group showed attenuated body mass loss in response to LPS as compared to the short-day group, despite exhibiting a longer duration of LPS-induced anorexia. It is possible that food restriction resulted in a slowing of metabolic rate in long-day restricted animals. Syrian hamsters (*Mesocricetus auratus*) recover from short-term food restriction by slowing down their resting metabolic rates, rather than increasing food consumption over normal pre-restriction levels (Borer et al., 1985). As Siberian hamsters also do not recover from energy deficit by increasing food consumption (Bartness and Clein, 1994), long-day restricted animals in this current study may have had reduced metabolic rates as well. Even though the long-day

restricted group may have shown greater intensity of LPS-induced anorexia than the short-day *ad libitum* group, if their metabolic rates were slower, they could have exhibited reduced body mass loss. The food restriction treatment may also explain why the long-day food restricted group displayed lesser decreases in nest building. Having access to a nest decreases food intake in Siberian hamsters housed in low temperatures by 18%, suggesting that nesting is an energy conservation strategy (Kauffman et al., 2003). Athough the long-day food restricted group was provided with access to 100% of their pre-restriction mean food intake, they may have still been in energy conservation mode, explaining why they did not decrease nest building as much as the two other groups.

In conclusion, our data show that food restricting long-day Siberian hamsters to mimic short-day body mass loss results in the attenuation of some sickness symptoms, rendering them more short-day like. However, our results do not provide conclusive evidence that all seasonal variation in sickness intensity can be attributed to seasonal changes in energy stores, as the intensity of some symptoms remained unchanged or intermediate between those of the short- and long-day *ad libitum* groups. One promising mediator of seasonal variation in sickness intensity may be glucocorticoids, as glucocorticoids act to suppress sickness and can vary both seasonally and with food restriction. Future work should target understanding the role of glucocorticoids in modulating seasonal sickness responses and work toward understanding how natural variation in energy stores within and across species and seasons contribute to sickness intensity.

CHAPTER 4: Glucose and insulin modulate sickness responses in male Siberian hamsters

Data in review at *General and Comparative Endocrinology*: Carlton, E.D. and Demas, G.E. Glucose and insulin modulate sickness responses in male Siberian hamsters.

Abstract

Mounting a sickness response is an energetically expensive task and requires precise balancing of energy allocation to ensure pathogen clearance while avoiding compromising energy reserves. Sickness intensity has previously been shown to be modulated by food restriction, body mass, and hormonal signals of energy reserves. In the current study, we tested the hypothesis that sickness intensity is modulated by glucose availability and an endocrine signal of glucose availability, insulin. We utilized male Siberian hamsters (*Phodopus sungorus*) and predicted that pharmacological induction of glucoprivation with 2-deoxy-D-glucose (2-DG), a non-metabolizable glucose analog that disrupts glycolysis, would attenuate energetically expensive sickness symptoms. Alternatively, we predicted that treatment of animals with insulin would enhance energetically expensive sickness symptoms, as insulin would act as a signal of increased glucose availability. Upon experimental treatment with lipopolysaccharide (LPS), we found that glucose deprivation resulted in increased sickness-induced hypothermia as compared to control- and insulin-treated animals; however, it did not have any effects on sickness-induced anorexia or body mass loss. Insulin treatment resulted in an unexpectedly exaggerated sickness response in animals of lesser body masses; however, in animals of greater body masses, insulin actually attenuated sickness-induced body mass loss and had no effects on hypothermia or anorexia. The effects

of insulin on sickness severity may be modulated by sensitivity to sickness-induced hypoglycemia. Collectively, these results demonstrate that both glucose availability and signals of glucose availability can modulate the intensity of energetically expensive sickness symptoms, but their effects differ among different sickness symptoms and are sensitive to energetic context.

Introduction

Animals must obtain and utilize energy to fuel virtually every physiological and behavioral process required for their survival and reproductive success. In times of energy limitation, constraints may require energy to be shunted away from processes of a lesser priority for an organism's current needs and toward those processes most fundamental for survival (Sheldon and Verhulst, 1996) . Mounting a sickness response is a process that requires precise balancing of energy allocation. Sickness responses are characterized by energetically expensive symptoms such as fever, anorexia, and body mass loss. These symptoms have adaptive benefits and reductions in their magnitude can negatively affect an animal's ability to clear its infection; however, if these symptoms are displayed too strongly, an animal may also succumb to death due to energy depletion (Adelman and Martin, 2009; Ashley and Wingfield, 2012; Hart, 1988; Moret and Schmid-Hempel, 2000).

Sickness responses are not static; animals are able to modulate sickness in response to their energy reserves. Previous work has shown that food restriction results in suppression of sickness symptoms (Bilbo and Nelson, 2002; MacDonald et al., 2014; MacDonald et al., 2011). Additionally, the intensity of the energetically

costly symptoms of sickness are correlated with body mass in several species, such that animals with higher body masses show more intense sickness responses (Carlton and Demas, in review; Owen-Ashley et al., 2008; Owen-Ashley et al., 2006; Pohl et al., 2014). Siberian hamsters (*Phodopus sungorus*) are a species that shows variation in sickness intensity that correlates with energetic state. For instance, hamsters show seasonal variation in both body mass and sickness intensity and exhibit the most intense sickness responses in the season in which they have the greatest body mass (Bilbo et al., 2002). We have previously manipulated body mass and an endocrine signal of fat stores (i.e., leptin) to determine their effects on sickness intensity variation in this species (Carlton and Demas, 2014; Carlton and Demas, in review). These studies showed that hamsters modulate sickness symptoms in response to decreases in energy stores (i.e., attenuation of sickness-induced anorexia and body mass loss in hamsters that were food restricted to lose body mass; Carlton and Demas, in review) and increases in circulating leptin levels (i.e., attenuation of sickness-induced hypothermia in hamsters provided exogenous leptin to simulate increased fat stores; Carlton and Demas, 2014).

In addition to signals of long-term energy stores, animals may also rely on signals of short-term energy availability (i.e., blood glucose levels) to modulate sickness. While an animal must avoid risking future survival by over-expending its excess energy stores, it may also be necessary for it to assess current environmental energy availability in order to avoid insufficient food resources during recovery. Glucose is the primary source of energy that is used by an animal, so blood glucose levels may provide the most immediate indicator of food availability. Furthermore,

glucose is critical for fueling immune responses (Wolowczuk et al., 2008). Previous work has shown that reductions in glucose availability via treatment with 2-deoxy-D-glucose (2-DG), a non-metabolizable glucose analog that disrupts glycolysis and induces a state of glucoprivation (Horton et al., 1973), impairs antibody production, delayed-type hypersensitivity responses, splenocyte production, and leukocyte counts in Siberian hamsters, deer mice (*Peromyscus maniculatus*), and Lewis rats (Chou et al., 1996; Demas et al., 1997b; Martin et al., 2008a; Zysling and Demas, 2007). Although there is considerable evidence that reducing glucose availability modulates many aspects of the immune response, it remains unclear how glucose availability may affect sickness responses.

While we can manipulate actual glucose availability with 2-DG, we can manipulate signals of glucose availability via the pancreatic peptide hormone insulin. Insulin is secreted upon food consumption, and its release facilitates the storage of energy. In the short-term, increased insulin levels signal positive energy balance, and levels rapidly change in response to an organism's current energetic state (Benoit et al., 2004). Insulin receptors are expressed on activated lymphocytes, and administration of insulin to these activated lymphocytes increases cellular metabolism (Delmastro-Greenwood and Piganelli, 2013; Helderman, 1981). Insulin has been shown to modulate immune responses in Siberian hamsters, specifically enhancing antibody production in the smaller short-day housed hamsters so that their antibody levels are comparable to those produced by the larger long-day housed hamsters (Garcia et al., 2010). These results suggest that

insulin may act as a signal of current energy availability to allow coordination of energetically-appropriate immune responses.

The goals of the present study were to manipulate current glucose availability and a signal of glucose to determine their effects on sickness response intensity in male Siberian hamsters. If glucose availability is a limiting factor in the display of energetically expensive sickness symptoms, then we expected that animals experiencing 2-DG-induced glucoprivation would show weakened fever or enhanced hypothermia and attenuated anorexia and body mass loss in response to the bacterial mimetic lipopolysaccharide (LPS) as compared to LPS-treated control animals. Similarly, we expected animals receiving insulin as a signal of increased glucose availability would show enhanced LPS-induced fever or weakened hypothermia and enhanced anorexia and body mass loss as compared to LPS-treated control animals. We also measured behaviors that are modulated during sickness in this species, thermoregulatory nest building behavior and hedonic behavior. We predicted that animals in the glucose-deprived group would show lesser sickness-induced declines in nesting behavior in response to LPS than the other two groups, as nesting can provide energy saving benefits; however, we predicted that we would see no differences in the decreases in hedonic behavior among the groups, as this behavior is not largely energetically demanding in this context.

Methods

Animals and housing conditions

Adult (> 60 days of age) male Siberian hamsters (n = 63) were obtained from our breeding colony at Indiana University. All animals were initially group housed (2-5 per cage with same sex siblings on weaning at 17-18 days of age) in long-day photoperiods (light:dark (L:D) 16:8) and then individually housed in polypropylene cages (27.8 x 17.5 x 13.0 cm) for one week prior to the start of the experiment. Animals were housed in long-day photoperiods for the entirety of the study. Food (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO, USA) and water were available *ad libitum* prior to and throughout the experiment. Temperature (20 ± 2°C) and humidity (50 ± 10%) were maintained at constant levels. All animal methods were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Indiana University.

Experimental methods

After one week of acclimation to individual housing, hamsters were quasi-randomly sorted (controlling for body mass, age, and genetic relatedness) into three groups: Control, 2-DG, or Insulin. Prior to receiving any experimental injections, daily measurements of body mass (to the nearest 0.1 g) and food consumption (to the nearest 0.1 g) were taken for five days. Food consumption was assessed by weighing the food pellets remaining in the hopper each day. Daily body mass and food consumption measurements continued through the entirety of the experiment.

After the five days of initial body mass and food intake measurements, hamsters started receiving daily injections. Animals in the Control group received one 0.2 ml intraperitoneal (i.p.) injection of 0.9% sterile saline every other day and one 0.1 ml subcutaneous (s.c.) injection of 0.9% sterile saline every day until the end of the experiment. Animals in the 2-DG group received one 0.2 ml i.p. injection of 1250 mg/kg 2-DG (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.9% sterile saline every other day and one 0.1 ml s.c. injection of 0.9% sterile saline every day until the end of the experiment. This dose was chosen because it is greater than the 2-DG dose that affects least one immune measure (i.e., antibody production) in this species but well below the dose that induces torpor (Dark et al., 1994; Zysling and Demas, 2007). The Insulin group received one 0.2 ml i.p. injection of 0.9% sterile saline every other day and one 0.1 ml s.c. injection of the long-lasting form of insulin, protamine zinc insulin (PZI) (ProZinc, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO, USA), dissolved in 0.9% sterile saline every day until the end of the experiment. The insulin dose was gradually increased over the course of 11 days to 20 U/kg, in order to avoid death-inducing severe hypoglycemia (Garcia et al., 2010). A dose of 20 U/kg was chosen because we wanted to modulate the signal of glucose availability without necessarily modulating actual blood glucose levels (Garcia et al., 2010). The PZI injections were administered according to the following dosing schedule: day 1: 0.625 U/kg; days 2-3: 1.25 U/kg; days 4-6: 5 U/kg; days 7-10: 10 U/kg; days 11-23: 20 U/kg. The 2-DG injections were provided on the odd-numbered experimental days.

On the 19th day of injections, a portion of the animals in each group were injected i.p. ~ 15 minutes before the onset of darkness (~1545 h) with 25 µg LPS (LPS from *Salmonella enterica* serotype typhimurium, Sigma-Aldrich, St. Louis, MO, USA; Carlton and Demas, 2014) suspended in 0.1 ml sterile 0.9% saline. Sickness responses were assessed throughout the four days following injections. The sample sizes were as follows: Control-Saline (n=10); Control-LPS (n=10); 2-DG-Saline (n=9); 2-DG-LPS (n=10); Insulin-Saline (n=11); Insulin-LPS (n=13).

Sickness response measurements

Following LPS injection, animals were monitored for signs of severe sickness, potentially indicating signs of sepsis, as outlined in our approved IACUC protocol. Animals that showed decreases in body temperature greater than 2°C below that of their group mean were removed from the study. These animals exhibited these temperature declines between 4 and 36 h post-injection. Due to a larger than expected number of animals showing these symptoms, we were able, post-hoc, to divide LPS-injected animals into two different groups: those that showed moderate sickness and remained in the study until the end (< 2°C decrease in colonic temperature) and those that showed severe sickness and were removed from the study (> 2°C decrease in colonic temperature).

Fever, anorexia, and body mass

On the day of LPS or saline injection, colonic temperatures (T_c; to the nearest 0.1 °C) were collected immediately before injection and 2, 4, 6, 8, 10, 12, 16, 20, and

24 h after injection using a MicroTherma 2T thermometer (ThermoWorks, Alpine, UT, USA) and a lubricated RET-3-ISO thermocouple probe (Physitemp Instruments, Inc., Clifton, NJ, USA) inserted ~12 mm into the rectum. To assess anorexia and body mass loss, daily body mass and food intake measurements continued until the end of the study.

Hedonic behavior

To assess the effects of our treatments on hedonic behavior, we provided hamsters with a highly palatable sodium saccharin solution (Baillie and Prendergast, 2008). Beginning 5 days before LPS or saline injection, for the first 6 h of the dark phase (1600 h to 2200 h) hamsters were provided with a fluid bottle containing a solution of 0.1% sodium saccharin (saccharin sodium salt hydrate, Sigma-Aldrich, St. Louis, MO, USA) dissolved in tap water (Baillie and Prendergast, 2008). The saccharin solution bottles were weighed (to the nearest 0.1 g) before they were given and after they were collected from the hamsters each day. Presentation of saccharin solution continued daily through day 3 post-injection.

Nest building behavior

To assess the effects of our treatments on thermoregulatory behavior, beginning five days before LPS or saline injection, each hamster was provided with a compressed cotton nestlet weighing ~2.5 g (Ancare, Bellmore, NY, USA) for the first 6 h of the dark phase (Baillie and Prendergast, 2008). The nestlet was weighed (to the nearest 0.1 g) before presentation, and the unshredded portion was weighed

after presentation. When provided a nestlet, hamsters quickly start shredding the cotton to build a nest. Nest building is an adaptive behavior to enhance energy conservation in low temperatures, however, hamsters readily build nests in room temperature (20-23 °C) (Puchalski et al., 1988). Presentation of nestlets continued daily through day 3 post-injection.

Blood sampling and necropsies

Blood samples were drawn from each animal 4 h after the onset of darkness (2000 h) at two time points (two days before injection and on the day of injection) to assess circulating blood glucose and serum cortisol concentrations. Briefly, animals were lightly anesthetized with isoflurane vapors, and blood samples were drawn from the retro-orbital sinus. Blood samples were allowed to clot at room temperature for 1 h, clots were removed, and samples were centrifuged at 4 °C for 30 min at 2500 rpm. Serum aliquots were aspirated and stored in sealable polypropylene microcentrifuge tubes at -20 °C until assayed. All blood samples were collected within 3 min of initial handling. Animals were euthanized five days after LPS injection and necropsies were performed. Testes, inguinal white adipose tissue (IWAT), epididymal WAT (EWAT), and retroperitoneal WAT (RWAT) were removed, cleaned of connective tissues, and weighed to the nearest 0.1 mg. A composite adipose tissue score was calculated by summing the individual WAT pad masses.

Blood glucose measurement

Blood glucose was measured from both blood samples. Immediately upon sampling, ~5 µl of whole blood was transferred onto the test strips of a blood glucose monitoring system (ReliOn, Micro Blood Glucose Monitoring System, Arkray USA, Inc., Minneapolis, MN, USA), and the readout was recorded. The meter was previously calibrated using an internal standard provided by the manufacturer.

Cortisol enzyme immunoassay (EIA)

We assessed circulating cortisol levels to determine if our treatments influenced the magnitude of LPS-induced hypothalamic-pituitary-adrenal (HPA) axis activation. Glucocorticoids are released during sickness and are important for regulating its intensity (Sapolsky et al., 2000). Cortisol is the predominant glucocorticoid in Siberian hamsters, with concentrations ~100x that of corticosterone (Reburn and Wynne-Edwards, 2000). Serum cortisol concentrations were determined in multiple enzyme immunoassays (EIAs) from a commercially prepared kit (Cortisol EIA Kit; Enzo Life Sciences, Inc., Farmingdale, NY, USA). This assay was previously validated for use in Siberian hamsters (Demas et al., 2004) and is highly specific for cortisol; cross-reactivity with corticosterone is 27.7% and < 4.0% for other steroid hormones. The sensitivity of the assay is 56.72 pg/ml. Samples were diluted 1:80 with assay buffer and run in duplicate. Intra-assay variabilities were 6.2%, 2.8%, and 3.1%.

Statistical analyses

All statistical tests were performed using JMP 10 (SAS Institute Inc., Cary, NC, USA). Residuals were checked for normality and homogeneity of variance, and those data that were non-normally distributed were transformed. Two animals were excluded from the final analyses: one from the LPS-treated 2-DG group which obtained an injury unrelated to the experimental procedures and one from the saline-treated Insulin group which exhibited sickness symptoms despite not receiving any LPS. The final sample sizes were as follows: Control-Saline (n=10); Control-LPS (n=10); 2-DG-Saline (n=9); 2-DG-LPS (n=9); Insulin-Saline (n=10); Insulin-LPS (n=13). Non-normally distributed variables differed in their degrees of skewness, so each variable was transformed with the function that best fit the data to normality. Baseline saccharin solution intake and percent change in saccharin solution intake were square root transformed, while cortisol concentrations were log transformed. Baseline percent nesting material shredded could not be transformed to meet the assumptions of normality, so a Kruskal-Wallis test was performed to determine if there were differences among the groups for this measure.

Pre-LPS baseline values were calculated for body mass, food intake, saccharin solution intake, and percent nesting material shredded by averaging the three daily measurements immediately prior to injections. To determine if there were effects of 2-DG or insulin treatment on pre-injection baseline body mass, baseline food intake, baseline saccharin solution intake, and pre-injection blood glucose levels, two-way (2-DG treatment (2) x insulin treatment (2)) analyses of

variance (ANOVA) were performed. Because expected values in some cells were less than five, Fisher's Exact Test was performed to determine if treatment affected the frequencies of animals within each LPS-treated group that had to be removed from the study due to severe sickness. Comparisons between animals that exhibited moderate sickness and continued in the study and those that exhibited severe sickness and were removed from the study were performed with two-sided t-tests. Animals that showed severe sickness and were removed from the study were not included in subsequent between group comparisons of post-injection percent changes in body mass, food intake, saccharin solution intake, and nesting material shredded, post-injection blood glucose concentrations, cortisol concentrations, and tissue masses (Results section 3.3).

Post-LPS changes in body mass, food intake, saccharin solution intake, and percent nesting material shredded were expressed as percentages of each animal's baseline values. Repeated measures ANOVAs were performed on post-injection percent changes in body mass, food intake, saccharin solution intake, and nesting material shredded and colonic temperature, with 2-DG, insulin, and LPS as between-subjects factors and time as the within-subjects factor. The within-subjects comparisons for percent change in saccharin solution intake and colonic temperature violated the assumptions of sphericity and were Greenhouse-Geisser corrected. Differences in post-injection glucose levels, cortisol concentrations, and tissue masses were assessed with three-way (2-DG (2) x insulin (2) x LPS (2)) ANOVAs. Post-hoc comparisons were conducted using Tukey's honestly significant difference (HSD) tests when ANOVAs were significant.

Results

Pre-LPS baseline measures

Before receiving any 2-DG or insulin treatments, the groups did not differ in body mass ($F_{2,58}$ = 0.88, P = 0.419) or food intake ($F_{2,58}$ = 0.25, P = 0.783). After treatments, all three groups showed decreases in body mass (T > 4.54, P < 0.001 for all comparisons), and the Insulin group showed increased food intake (T = 2.66, P = 0.014) while the Control and 2-DG groups showed no change in food intake pre- to post-treatment (T < 0.97, P > 0.347). After treatments and before LPS or saline injection, baseline body mass ($F_{2,58}$ = 1.39, P = 0.256), baseline saccharin solution intake ($F_{2,58}$ = 2.03, P = 0.140), baseline percent nesting material shredded (H = 4.07, P = 0.131), and pre-injection blood glucose levels ($F_{2,58}$ = 1.82, P = 0.171) did not differ among the Control, 2-DG and Insulin groups. Alternatively, pre-injection baseline food intake was affected by insulin treatment ($F_{1,58}$ = 6.20, P = 0.016) (Table 1). Specifically, the Insulin group showed greater daily food intake than the Control group (P = 0.041). With the exception of baseline food intake in the 2-DG group (i.e., LPS-injected hamsters exhibited greater baseline food intake values than saline-injected hamsters; T = 2.49, P = 0.025), none of the baseline measures differed between LPS- and saline-injected hamsters within each group (P > 0.085 for all comparisons).

Table 1. Mean (± SEM) baseline body mass, food intake, saccharin solution intake, percent nesting material shredded, and blood glucose levels prior to LPS or saline injection in control, 2-DG, and insulin-treated male Siberian hamsters. Baseline measurements were calculated by averaging the values collected for the measurements during the three days prior to LPS or saline injection. Groups with different letters indicate statistically significant differences between group means ($P < 0.05$); groups sharing the same letter (or no letters) are statistically equivalent.

	Control	**2-DG**	**Insulin**
Baseline Body Mass (g)	43.9 ± 1.4	45.7 ± 1.2	47.0 ± 1.3
Baseline Food Intake (g/day)	4.7 ± 0.1[a]	4.9 ± 0.2[a,b]	5.2 ± 0.1[b]
Baseline Saccharin Solution Intake (g / 6 h)	1.6 ± 0.2	1.8 ± 0.2	2.2 ± 0.2
Baseline Percent Nesting Material Shredded	93.7 ± 2.7	91.6 ± 4.7	83.6 ± 4.3
Pre-injection blood glucose concentration (mg/dl)	107 ± 4	98 ± 5	96 ± 4

Animals displaying severe sickness

After LPS-injection, six of the 13 animals in the Insulin group exhibited severe sickness and were removed from the study. No LPS-treated animals from the Control or 2-DG groups were removed from the study. The frequencies of animal removal among the three LPS-treated groups were significantly different (Fishers Exact Test, $P = 0.005$). Within the LPS-injected Insulin group, those animals that exhibited severe sickness showed lower baseline body masses ($T = -5.29$, $P < 0.001$), higher pre-injection blood glucose levels ($T = 2.41$, $P = 0.035$), lower post-injection blood glucose levels ($T = -2.83$, $P = 0.018$), and higher post-

injection cortisol concentrations ($T = 2.92$, $P = 0.014$) as compared to animals that exhibited moderate sickness (Table 2). There were no differences between the moderate and severe sickness animals in baseline food intake, baseline saccharin solution intake, baseline percent nesting material shredded, or colonic temperature immediately prior to injection ($P > 0.063$ for all comparisons).

Table 2. Mean (± SEM) baseline body mass, food intake, saccharin solution intake, percent nesting material shredded and colonic temperature and blood glucose levels prior to LPS injection and blood glucose and serum cortisol levels 4 h after LPS injection in insulin-treated male Siberian hamsters that exhibited moderate or severe sickness symptoms. Hamsters were classified as exhibiting moderate sickness and kept in the study if they showed a decrease in colonic temperature < 2°C from the group mean after LPS injection and as severe sickness and removed from the study if they showed a decrease in colonic temperature > 2°C from the group mean after LPS injection. *$P < 0.05$ and **$P < 0.001$ for differences between the two groups.

Measure	Moderate	Severe
Baseline Body Mass (g)	52.5 ± 1.2	40.1 ± 2.0**
Baseline Food Intake (g/day)	5.6 ± 0.1	4.8 ± 0.3
Baseline Saccharin Solution Intake (g/6h)	2.9 ± 0.5	2.1 ± 0.5
Baseline Percent Nesting Material Shredded	88.1 ± 5.8	70.7 ± 12.3
Pre-injection Colonic Temperature (°C)	37.1 ± 0.2	36.7 ± 0.2
Pre-injection Blood Glucose (mg/dl)	85 ± 8	112 ± 8.0*
Post-injection Blood Glucose (mg/dl)	51 ± 5	31 ± 5*
Post-injection Cortisol (ng/ml)	134.6 ±12.9	189.3 ± 13.6*

Sickness, physiological, and morphological measurements in animals showing moderate sickness responses

Colonic temperature

Post-injection colonic temperature was affected by LPS injection ($F_{1,49}$ = 16.08, $P < 0.001$) but not by any of the other between subjects factors or between subjects interactions ($P > 0.29$ for all effects) (Figure 1). Temperature did change across time (within subjects, $F_{5.4,265.9} = 50.78$, $P < 0.001$, G-G corrected) and with the time x LPS ($F_{5.4,265.9} = 8.48$, $P < 0.001$, G-G corrected) and time x 2-DG ($F_{5.4,265.9} = 3.13$, $P = 0.008$, G-G corrected) interactions. Specifically, all LPS-injected groups showed varying degrees of hypothermia post-injection. The LPS-injected 2-DG group exhibited temperatures lower than their respective saline-injected controls at the greatest number of timepoints post-injection (6, 10, 16, 20, and 24 h); whereas the LPS-treated Control and LPS-treated Insulin groups only showed lower temperatures than their saline-injected controls at two (6 and 16 h) and one (6 h) timepoints post-injection, respectively.

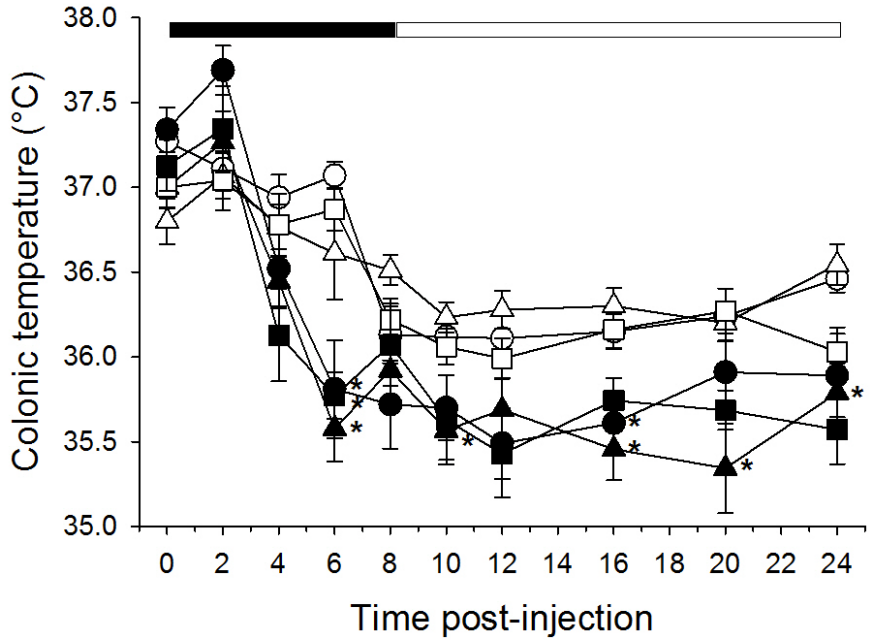

Figure 1. Mean (± SEM) colonic temperature following LPS (black icons) or saline (white icons) injection delivered at the 0 h timepoint in control- (●), 2-DG- (▲), or insulin- (■) treated male Siberian hamsters. Black and white bars at the top of the graph indicates the active, dark (black) and inactive, light (white) phases of the light-dark cycle. $^{*}P < 0.05$ versus saline-injected group given the same treatment.

Anorexia

Percent change in food intake was affected by LPS injection ($F_{1,48} = 98.82$, $P < 0.001$) but not by any of the other between subjects factors or between subjects interactions ($P > 0.27$ for all effects) (Figure 2A). In addition, percent changes in food intake changed across time (within subjects, $F_{3,46} = 101.81$, $P < 0.001$) and with the time x LPS ($F_{3,46} = 11.61$, $P < 0.001$) and time x insulin ($F_{3,46} = 4.44$, $P = 0.008$) interactions. Specifically, all LPS-injected groups showed greater percent decreases in food intake in comparison to their respective saline-injected controls at days 1, 2, and 3 post-injection ($P < 0.05$ for all comparisons). By day 4 post-injection, all LPS-injected groups no longer showed changes in food intake that

differed from their respective saline-injected controls ($P > 0.09$ for all comparisons).

Body mass loss

Percent change in body mass loss was affected by LPS injection ($F_{1,49} = 102.47$, $P < 0.001$), 2-DG treatment ($F_{1,49} = 4.25$, $P = 0.045$), insulin treatment ($F_{1,49} = 4.29$, $P = 0.044$) and the insulin x LPS interaction ($F_{1,49} = 6.78$, $P = 0.012$) but not the 2-DG x LPS interaction ($F_{1,49} = 0.25$, $P = 0.618$) (Figure 2B). In addition, body mass loss changed with the time x LPS interaction (within subjects, $F_{3,47} = 14.15$, $P < 0.001$) but not with any other of the within subjects factors or interactions ($P > 0.08$ for all tests). Specifically, LPS-injected Insulin animals showed attenuated body mass loss as compared to LPS-injected Control and 2-DG animals. Whereas the LPS-injected Control and 2-DG groups showed greater percent decreases in body mass loss in comparison to their respective saline-injected groups at all four timepoints post-injection ($P < 0.001$ for all comparisons), the LPS-injected Insulin group only showed greater percent decreases in body mass loss in comparison to its saline-injected control group at days 2, 3, and 4 post-injection ($P = 0.068$ on day 1, $P < 0.001$ on other days). Furthermore, the LPS-injected Insulin group showed attenuated post-LPS body mass loss compared to the LPS-injected 2-DG group at day 2 post-injection and both the LPS-injected 2-DG and Control groups at day 3 post-injection ($P < 0.005$ for these comparisons).

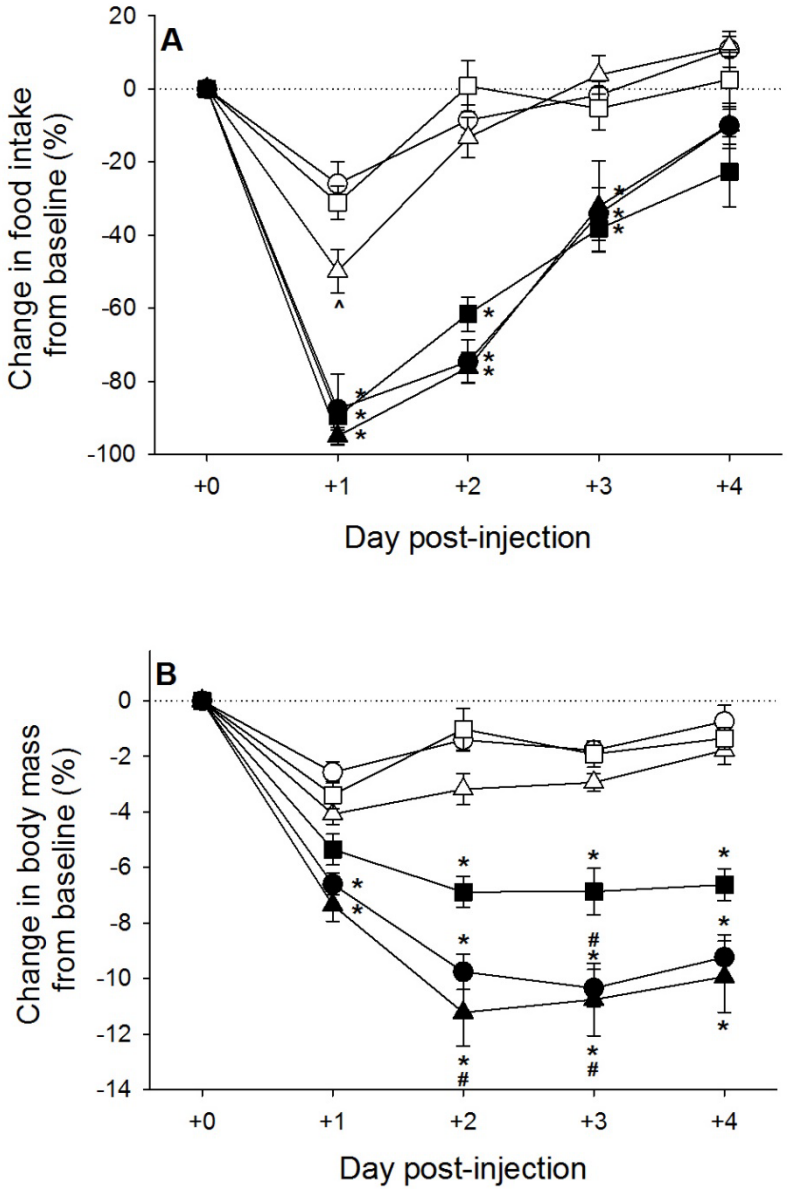

Figure 2. Mean (± SEM) percent changes in (A) food intake and (B) body mass from baseline following LPS (black icons) or saline (white icons) injection delivered on day 0 in control- (●), 2-DG- (▲), or insulin- (■) treated male Siberian hamsters. Day +1 represents the time period from 0-24 h after LPS or saline injection, Day +2 represents the time period from 24-48 h after LPS or saline injection, and so forth. Within each panel: $^{*}P < 0.05$ versus saline-injected group given the same treatment. $^{\#}P < 0.05$ versus LPS-injected Insulin group. $^{\wedge}P < 0.05$ versus other saline-injected groups.

Saccharin solution intake

Percent change in saccharin intake after injection was affected by LPS injection ($F_{1,49}$ = 7.34, P < 0.009) but not by any of the other between subjects factors or between subjects interactions (P > 0.43 for all effects) (Figure 3A). Furthermore, saccharin solution intake changed with time (within subjects, $F_{2.3,110.7}$ = 11.69, P < 0.001, G-G corrected) and with the time x LPS interaction ($F_{2.3,110.7}$ = 2.23, P = 0.001, G-G corrected). Specifically, at the 0-6 h timepoint, animals in the LPS-injected 2-DG and Insulin groups showed greater percent decreases in saccharin solution intake as compared to their respective saline-injected controls (P < 0.044 for both comparisons), while there was no difference between the LPS-injected Control group and its respective saline-injected control (P = 0.098).

Nesting material shredded

Percent change in nesting material shredded after injection was affected by LPS injection ($F_{1,49}$ = 188.82, P < 0.001) but not by any of the other between subjects factors or between subjects interactions (P > 0.09 for all effects) (Figure 3B). Percent nesting material shredded did change over time (within subjects, $F_{3,47}$ = 41.18, P < 0.001) and with the time x LPS interaction (within subjects, $F_{3,47}$ = 56.30, P < 0.001). Specifically, animals from all LPS-injected groups showed greater percent decreases in nesting material shredded than their respective saline-injected controls at the 0-6 h, 24-30 h, and 48-54 h timepoints (P < 0.040 for all comparisons); nest shredding returned to levels similar to saline-injected controls by the 72-78 h timepoint (P > 0.998 for all comparisons). In addition, animals in

the LPS-injected 2-DG group showed a greater percent decrease in nesting material shredded in comparison with the LPS-injected Control group at the 48-54 h timepoint ($P = 0.016$).

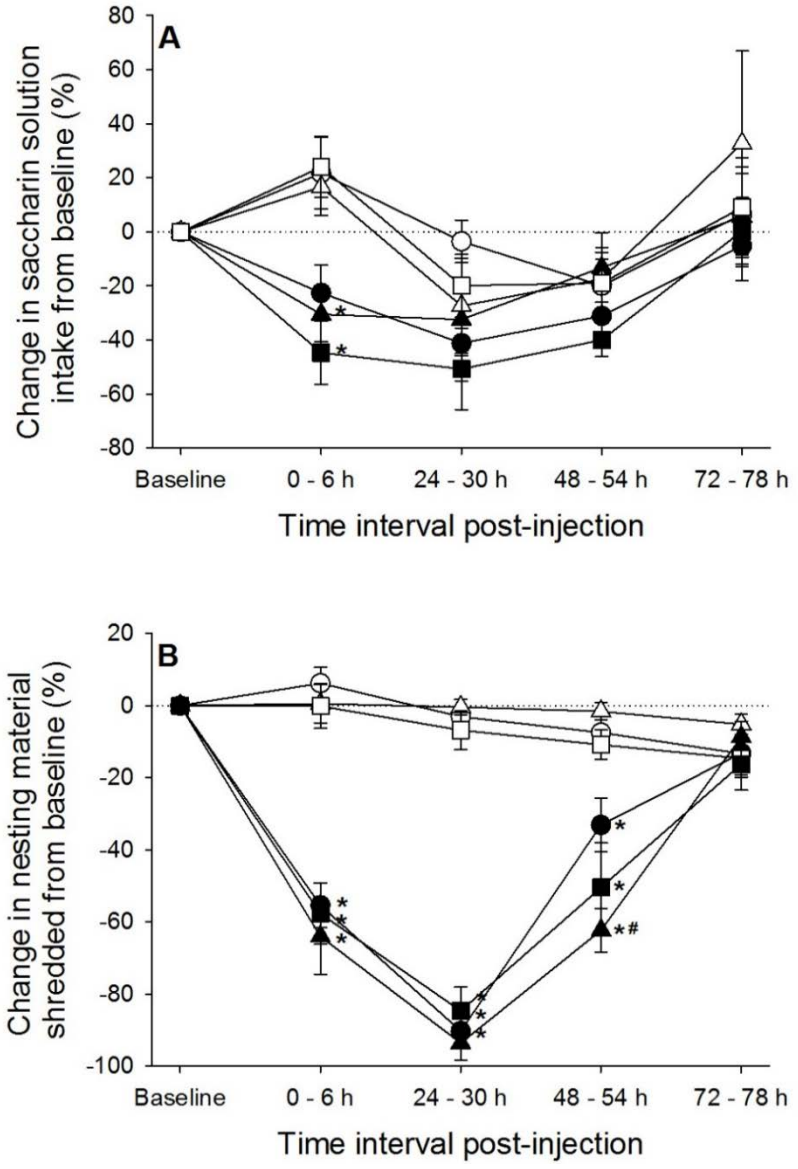

Figure 3. Mean (± SEM) percent changes in (A) saccharin solution intake and (B) percent nesting material shredded from baseline following LPS (black icons) or saline (white icons) injection delivered at the 0 h timepoint in control- (●), 2-DG- (▲), or insulin- (■) treated male Siberian hamsters. $^{*}P < 0.05$ versus saline-injected group given the same treatment. $^{\#}P < 0.05$ versus LPS-injected Control group.

Blood glucose

Post-injection blood glucose concentrations were affected by LPS injection ($F_{1,49}$ = 35.37, P < 0.001) and insulin treatment ($F_{1,49}$ = 7.18, P = 0.010) but not by 2-DG or the LPS x insulin and LPS x 2-DG interactions (P > 0.47 for all effects) (Figure 4A). Specifically, all LPS-injected groups had lower blood glucose concentrations than their respective saline-injected controls (P < 0.02 for all comparisons), and insulin-treated animals, as a whole, had lower post-injection glucose levels as compared to animals not treated with insulin (T = -2.77, P =0.008).

Serum cortisol

Post-injection serum cortisol concentrations were affected by LPS injection ($F_{1,49}$ = 40.19, P < 0.001) but not by 2-DG or insulin treatment or their interactions with LPS (P > 0.37 for all effects) (Figure 4B). Specifically, all LPS-injected animals showed greater cortisol levels than their respective saline-injected controls (P < 0.004 for all comparisons).

Tissue masses

Paired testes mass was affected by 2-DG treatment ($F_{1,49}$ = 6.62, P = 0.013) but not by LPS or insulin treatment or any interactions (P > 0.321 for all effects). Specifically, as a whole, animals treated with 2-DG had lesser paired testes masses as compared to animals not treated 2-DG (0.45 g ± 0.06 SE vs. 0.63 g ± 0.03 SE, respectively) (T = -2.57, P =0.013). Composite adipose tissue mass was not affected by any of the treatments or interactions ($F_{5,49}$ = 0.842, P = 0.526).

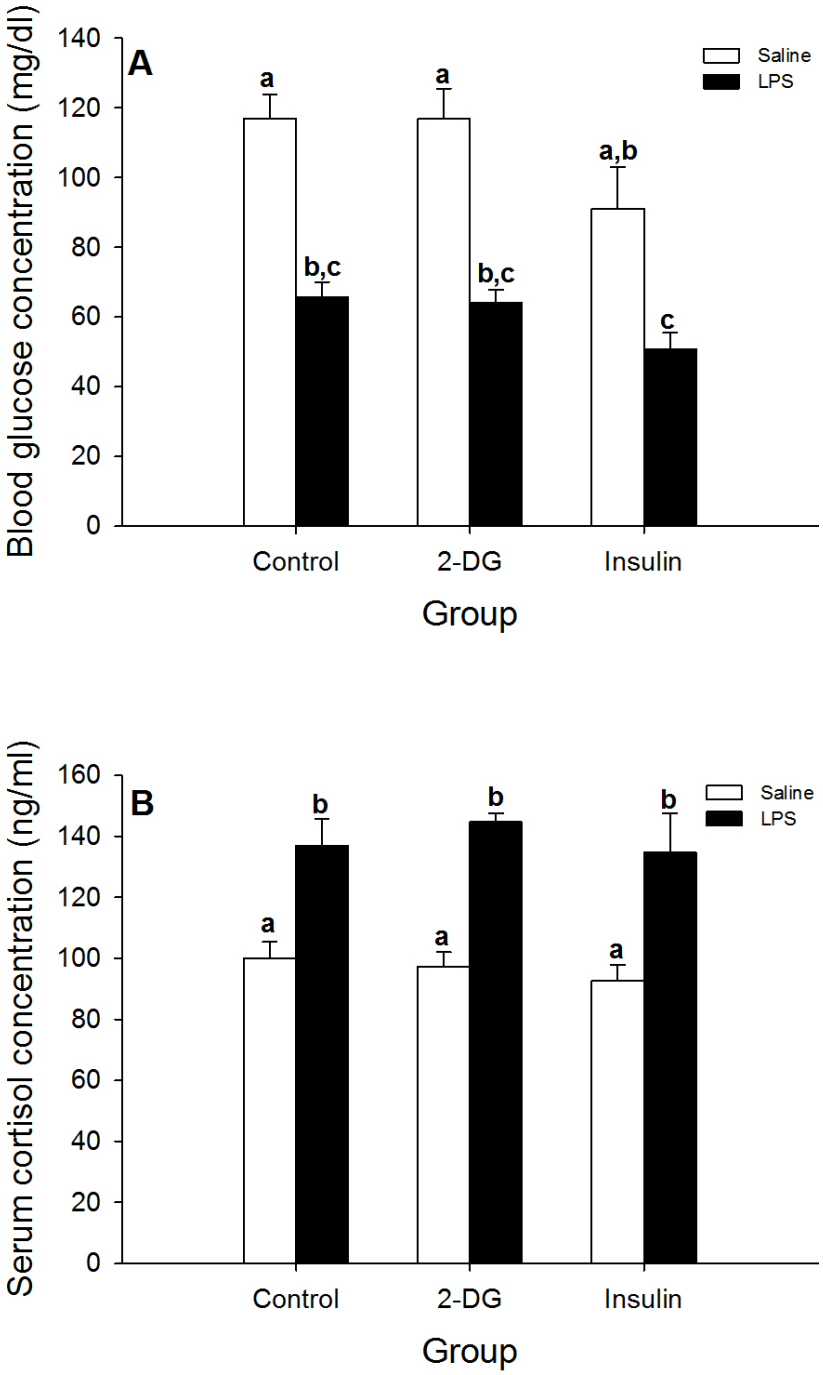

Figure 4. Mean (± SEM) (A) blood glucose and (B) circulating serum cortisol concentrations taken 4 h following LPS or saline injection in control-, 2-DG-, or insulin-treated male Siberian hamsters. Within each panel, groups with different letters indicate statistically significant differences between group means ($P < 0.05$); groups sharing the same letter are statistically equivalent.

Discussion

The results of this study demonstrate that both direct manipulations of glucose availability, as well as signals of glucose availability, modulate the intensity of some of the energetically expensive symptoms of sickness. Specifically, reductions in glucose availability in the 2-DG group resulted in a greater hypothermic response as compared to the other two groups, potentially as an energy saving mechanism. Alternatively, contrary to our predictions, animals experiencing reduced glucose availability showed a more pronounced sickness-induced decrease in nesting behavior as compared to control animals. Furthermore, among the animals that showed moderate sickness, there were no treatment effects on sickness-induced anorexia and contrary to our predictions, insulin-treated animals showed an attenuation of sickness-induced body mass loss as compared to the other two groups.

In addition to these effects on specific sickness symptoms, we also saw that a portion of the animals that were provided signals of increased glucose availability via insulin treatment displayed severe sickness after LPS injection. Although all insulin-treated animals showed hypoglycemia after LPS injection, the insulin-treated animals that exhibited severe sickness showed lower glucose levels post-injection than animals exhibiting moderate sickness. This relationship between post-LPS glucose levels and severe sickness is consistent with prior work in rats that showed that severe endotoxemia is associated with greater hypoglycemia (Lang et al., 1985). Although it seems that the drop in blood glucose may be predictive of which animals showed severe sickness, it is still not clear why the

animals that exhibited severe sickness were also the animals with the lowest body masses in the insulin-treated group. One possible explanation is that these smaller individuals had increased sensitivity to sickness-induced hypoglycemia due to differences in LPS dosing. Even though the insulin doses were corrected for body mass, all animals received a standard dose of LPS (25 µg). Therefore, smaller animals received a higher dose of LPS relative to their body masses. As all LPS-injected groups were matched for body mass prior to injection, it is not likely that this severe sickness response was only due to dose differences among smaller and larger animals, as none of the smaller animals from the Control and 2-DG groups showed severe sickness. Therefore, it appears that insulin treatment may have increased the sensitivity of animals to LPS treatment.

Interleukin (IL)-1, a pro-inflammatory cytokine released in response to LPS and infection, affects glucose homeostasis during infection and induces hypoglycemia, as it modulates glucose regulation via the brain and stimulates increases in glucose utilization by peripheral tissues (del Rey and Besedovsky, 1989; del Rey et al., 2006; Vogel et al., 1991). While animals in the current study exhibited hypoglycemic responses to LPS treatment, it is also common for human and non-human animals to exhibit hyperglycemic responses to infection (McGuinness, 2005). In cases of sickness-induced hyperglycemia, insulin treatment improves survival in the critically ill, and this is likely mediated through a normalization of glucose levels via decreases in pro- and increases in anti-inflammatory cytokine release (Jeschke et al., 2004; van den Berghe et al., 2001). In the current study, all LPS-treated animals showed hypoglycemia, so

administering insulin during experimental infection likely was the cause of the potentiated hypoglycemic response in the insulin-treated animals as compared to the other two groups. Thus, the potential enhanced hypoglycemic effects of the greater LPS dose in these smaller animals coupled with the hypoglycemic effects of insulin treatment may explain why the smaller animals in the insulin-treated group showed the most severe sickness responses of all groups.

Whereas insulin treatment facilitated an enhanced sickness response in the smaller individuals in that group, the larger individuals that exhibited moderate sickness and continued in the study actually showed attenuation of an energetically expensive sickness symptom. The insulin-treated animals that remained in the study showed lesser percent decreases in body mass after LPS injection as compared to the Control and 2-DG groups. This attenuation of body mass loss in the insulin-treated group is not a product of differences in sickness-induced anorexia, as all three LPS-treated groups showed similar patterns and intensities of anorexia post-LPS. Furthermore, this attenuation is surprising because the animals that displayed moderate sickness in the LPS-treated Insulin group had an average body mass that was 6-8 grams greater than animals in the LPS-treated Control and 2-DG groups. In Siberian hamsters and white-crowned sparrows (*Zonotrichia leucophrys*), pre-sickness body mass is negatively correlated with percent decrease in body mass loss after LPS-injection, such that larger individuals show greater percent decreases in body mass after injection, even when all animals are given the same quantity of LPS (Carlton and Demas, in review; Owen-Ashley et al., 2008; Owen-Ashley et al., 2006). Based upon these previous results, it was expected that

the LPS-injected Insulin group would show even greater percent decreases in body mass loss than we initially predicted (i.e., when we expected that all LPS-treated groups would share similar average body masses). Although we did not see insulin attenuate sickness responses in the smaller individuals in the group, these heavier animals that continued until the end of the study could have experienced some of the anti-inflammatory properties of insulin (Jeschke et al., 2004). The LPS-injected Insulin group showed attenuated body mass loss compared to the other groups at days 2 and 3 post-LPS. At this point in the study, if animals were no longer hypoglycemic, insulin-treatment could have induced anti-inflammatory mechanisms rather than exacerbating hypoglycemia as it did in the few hours after LPS injection.

Animals experiencing glucose deprivation showed the most pronounced hypothermic responses of the three LPS-treated groups. Hypothermic responses to LPS treatment or infection, rather than febrile responses, are hypothesized to be advantageous for pathogen clearance when energy availability is low or in cases where animals are experiencing more severe inflammatory responses (Deen and Hutchison, 2001; Romanovsky and Szekely, 1998). In the context of this study, the hypothermic response exhibited by all LPS-treated groups is more likely a response to greater inflammation rather than energy availability, as animals in the Control and Insulin groups were experiencing no sources of energy limitation. Alternatively, the 2-DG group was experiencing energy limitation, and the increased hypothermic response shown by this group could have been a mechanism of energy conservation. In a previous study, we showed that hamsters that were

experimentally provided with a hormonal signal of increased energy stores (i.e., leptin) showed lesser LPS-induced hypothermic responses as compared to animals receiving a vehicle control (Carlton and Demas, 2014). The results of the current study further support the conclusion that the magnitude of LPS-induced hypothermia can be modulated by energetic state and signals of energetic state, as hypothermia was enhanced when animals were experimentally deprived of glucose.

While 2-DG disrupts glycolysis and induces glucoprivation, it is often used in experimental contexts as a "metabolic stressor," as its effects on glucose use are often accompanied by activation of the HPA axis and increased circulating glucocorticoid levels (Demas et al., 1997b; Weidenfeld et al., 1994). In the current study, there were no effects of 2-DG on cortisol levels in both the saline- and LPS-injected animals. This lack of increased cortisol in response to 2-DG has been observed in two other studies in this species, so it was not necessarily unexpected (Carlton et al., 2014; Zysling and Demas, 2007). Glucocorticoids are critical for the regulation of inflammatory responses and sickness symptoms (Sapolsky et al., 2000). Specifically, lack of glucocorticoid regulation of the inflammatory response during infection can augment the amplitude and duration of fever, enhance behavioral depression, and in some cases, increase susceptibility to death; however, glucocorticoid replacement can ameliorate these effects (Coelho et al., 1992; Goujon et al., 1995a; Morrow et al., 1993; Ruzek et al., 1999). Glucocorticoids are also involved in the regulation of sickness-induced hypothermia (Goujon et al., 1995b); however, since there were no treatment effects on either saline- or LPS-injected cortisol levels, this suggests that the increased hypothermic response in the

LPS-injected 2-DG group was mediated by changes in energy availability rather than glucocorticoids produced as a product of metabolic stress.

One reason why we may not have seen attenuation of sickness-induced anorexia, body mass loss, or percent decreases in nesting material shredded in the LPS-injected 2-DG group was that animals in this group were shifting energy away from other physiological systems in order to allocate energy to the immune system. 2-DG-treated animals had lower paired testes masses than the animals in the Control and Insulin groups, which suggests that animals in this group were shifting energy away from reproductive maintenance. Previous work has found that when experiencing glucose deprivation while mounting a delayed-type hypersensitivity (DTH) immune response, male Siberian hamsters show reduced testes mass but no reductions in the magnitude of their DTH response as compared to animals not experiencing glucose deprivation (Martin et al., 2008a). In females, the reverse is found, as glucose-deprived animals show no reductions in reproductive tissue masses but show decreases in DTH response as compared to controls (Martin et al., 2008a). Thus, in the current study, if energy had not been shifted away from reproductive maintenance in 2-DG-treated animals, then we may have seen attenuation of these additional energetically-expensive sickness symptoms in this group.

In conclusion, our data show that sickness symptoms in Siberian hamsters can be modulated by glucose deprivation and by signals of increased glucose availability. Insulin treatment appears to have both enhancing and suppressive effects on sickness in this species, as it facilitated severe sickness responses in

smaller individuals but attenuated sickness-induced body mass loss in the larger individuals that displayed moderate sickness. Glucose deprivation resulted in increased sickness-induced hypothermia, but its lack of effect on the other energetically expensive sickness symptoms suggests that energy may have been reallocated from another physiological system (i.e., the reproductive system) to maintain the full expression of sickness symptoms in this group. Collectively, these results suggest that glucose availability can modulate sickness symptoms but that its effects depend on factors such as infection severity and energetic trade-offs among multiple physiological systems.

CONCLUSIONS

Allocation of finite energy resources to competing physiological and behavioral processes is essential for ensuring survival and reproductive success in both predictable and unpredictable environmental conditions. Seasonally-breeding animals experience predictable changes in environmental energy availability as the seasons change between the more resource-abundant spring and summer months to the less energetically-plentiful fall and winter months. Alternatively, within seasons, unexpected variation in abiotic or biotic factors can result in periods of resource abundance or scarcity. Internally, animals have a suite of endocrine and neuroendocrine factors that respond to changes in food consumption and existing energy reserves and act to regulate energy allocation among different processes. **The broad goal of this dissertation was to elucidate the function of different forms of energy and their signals on the regulation of energy allocation to immune function and sickness responses within and across seasons in Siberian hamsters (Figure 1).** To accomplish this goal, I assessed the roles of two main forms of chemical energy used by animals (i.e., glucose and fat) and their signals (i.e., insulin and leptin) in energy allocation. I experimentally manipulated glucose availability via pharmacological induction of glucoprivation with the non-metabolizable glucose analog 2-deoxy-D-glucose (2-DG) and modulated signals of glucose availability with treatment with the pancreatic peptide hormone insulin. I manipulated fat reserves through long-term food restriction and altered signals of fat reserves with treatment with the adipose peptide hormone leptin. Use of these

manipulations, individually or simultaneously, allowed me to tease apart the mechanisms of energy allocation toward and away from immune responses.

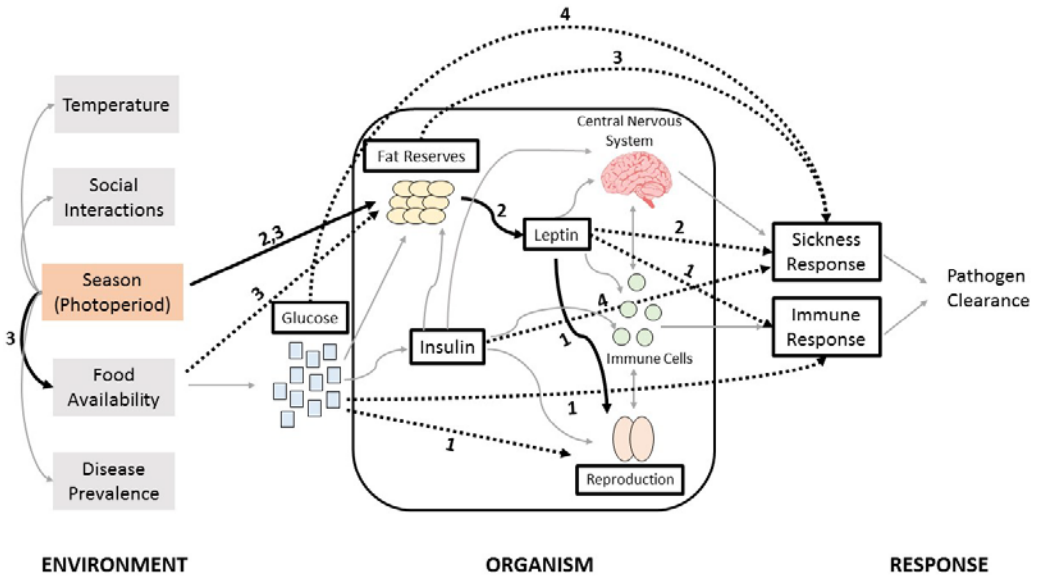

Figure 1. A simplified model illustrating the measured and unmeasured environmental, physiological, and immunological variables assessed in this dissertation. Briefly, environmental variables affect energy intake (i.e., glucose) and reserves (i.e., fat) of an organism. These environmental variables can all change predictably and simultaneously with the seasons. Alterations in glucose intake and fat reserves result in changes in circulating levels of the hormones insulin and leptin, respectively. These hormones increase with increased glucose concentrations or fat masses and decrease with lower quantities of these energy sources. Insulin and leptin can act on multiple tissues and systems, including the reproductive, immune, and nervous systems (and energy prioritized to one of these systems can result in lesser energy allocation to another system). Immune responses are generated by activated immune cells and sickness responses are generated via a coordination between immune cells and the brain. Finally, the immune and sickness responses generated by the organism result in clearance of the pathogen. Black boxes around text highlight variables that were measured and/or manipulated in this dissertation. Solid black lines indicate direct pathways between variables that were manipulated and quantified in this dissertation. Dotted black lines indicate indirect pathways between variables that were manipulated and quantified in this dissertation (i.e., some intermediaries in the direct pathway were not investigated or quanitified). Grey lines show pathways that exist between variables but were not assessed in these studies. The numbers found along the solid or dotted black lines refer to the dissertation chapter in which the link was measured or manipulated.

Summary

The purpose of Chapter 1 was to determine how different severities of glucose deprivation affect energy allocation between the immune and reproductive systems and if the effects of glucose limitation on these trade-offs can be alleviated with a neuroendocrine signal of increased fat stores. I demonstrated that energy allocation in reproductively-active female Siberian hamsters depends on the severity of glucose deprivation, as animals experiencing lower levels of glucose deprivation allocated energy away from reproductive cycling, however, animals experiencing more severe glucose deprivation maintained reproductive cycling. Providing a signal of increased fat stores via leptin administration alleviated the reproductive inhibition in animals experiencing lower levels of glucose deprivation, suggesting that under mild energetic stress, tapping into a different energetic system (i.e., lipid stores) can alleviate the suppressive effects of this stress. Alternatively, experiencing high levels of glucoprivation while receiving a signal of increased fat stores resulted in lower antibody production, possibly indicating that resources were shifted toward another unmeasured physiological system or process in this case.

The purpose of Chapter 2 was to examine if seasonal variation in sickness intensity is mediated by seasonal changes in circulating leptin levels. Since I discovered in Chapter 1 that leptin altered energy allocation toward humoral immunity, I wanted to determine its role as an energetic signal that could modulate seasonal sickness intensity. I found that providing short-day housed male Siberian hamsters with exogenous leptin to simulate long-day leptin levels modulated

sickness-induced hypothermia, such that it was attenuated in this group as compared to vehicle-treated short-day hamsters. This result suggests that seasonal variation in leptin levels may mediate the magnitude of sickness-induced body temperature changes. Contrary to predictions, sickness-induced anorexia was attenuated, rather than enhanced, in short-day leptin-treated animals, indicating that seasonal changes in leptin levels do not modulate seasonal sickness intensity as a whole. Rather, different sickness symptoms may be coordinated by other energetic (or non-energetic) signals.

The purpose of this Chapter 3 was to determine if seasonal variation in sickness intensity was modulated by seasonal changes in body mass. The lack of leptin-induced modulation of the full suite of sickness symptoms in Chapter 2 led us to investigate if seasonal decreases in actual energy stores would facilitate attenuation of the sickness response as a whole. I used experimental food restriction to induce a short-day-like pattern of body mass loss in long-day housed hamsters. I predicted that if seasonal changes in body mass modulate seasonal variation in sickness responses, then long-day food-restricted animals would show short-day-like sickness responses. I discovered that long-day food restricted animals showed more short-day-like body mass loss, anorexia, and hypothermia in response to sickness, suggesting that seasonal changes in body mass may mediate some of the variation in sickness intensity across the seasons. However, the anorexic and hypothermic responses of the long-day food-restricted group fell intermediate between those of the short-day and long-day *ad libitum* groups, even though there were no differences in body mass between the long-day food-restricted and short-

day *ad libitum* groups. These results suggest the possibility that seasonal changes in circulating cortisol levels may be involved in the attenuation of sickness symptoms in short-day animals, providing a new avenue for future research in the mechanisms coordinating seasonal variation in sickness intensity.

The purpose of Chapter 4 was to determine how sickness intensity in long-day conditions is modulated by glucose availability and a signal of glucose availability. In Chapters 2 and 3, I manipulated energy stores and a signal of energy stores, simulating predictable seasonal energetic changes for the organism. In Chapter 4, I wanted to investigate how sickness intensity is modulated in response to more short-term and unpredictable changes in glucose availability. I found that glucose deprivation resulted in increased sickness-induced hypothermia, yet had no effects on sickness-induced anorexia and body mass. This result suggests that glucose availability does limit the magnitude of at least one energetically-expensive sickness symptom in long-day animals but not all symptoms. Insulin treatment had opposing effects on sickness intensity depending on the pre-sickness body mass of the individual. Smaller animals showed severe responses to LPS treatment, requiring their removals from the study, whereas larger animals actually showed attenuated LPS-induced body mass loss. The presence of lower blood glucose levels after LPS injection in the smaller animals that displayed severe sickness as compared to the larger animals that displayed moderate sickness suggests that insulin may have severe potentiating effects on sickness-induced hypoglycemia in smaller animals.

Implications and future directions

These present research findings highlight the complexity of determining the effects of different energetic signals and stressors on physiological and behavioral responses. In this dissertation, I focused my efforts on understanding how two energy sources and two neuroendocrine signals of energy availability influence reproductive-immune trade-offs and sickness intensity. One of the recurring themes throughout this dissertation is that these energy sources and their signals cannot be modulated in a simple input-output system, but instead, changes in these individual sources and signals result in feedback and responses that lead to unexpected energetic trade-offs. This result is not necessarily surprising as, highlighted in Figure 1, leptin and insulin do not only act on the immune system but also have effects on the nervous and reproductive systems, in addition to other physiological systems not included in this simplified model. Thus, even though I only measured two main physiological systems in this dissertation, it is unlikely that these are the only two systems that are responding to my manipulations of energy source and signal. Furthermore, the endocrine and neuroendocrine signals involved in energy regulation are numerous and responsive to each other (Badman and Flier, 2005; Spiegelman and Flier, 2001). Therefore, when we manipulate one signal, we are likely manipulating a suite of other factors involved in energy regulation.

Throughout this dissertation, I found examples of these complex interactions between energy sources and signals. These examples were often manifested as unpredicted occurrences of or lacks of energetic trade-offs. Trade-offs were observed throughout all four chapters and at all different levels of

investigation—between fuel sources, within immune responses, and across different physiological systems. Examples of trade-offs between fuel sources were observed in Chapters 1 and 3. For instance, in Chapter 1, contrary to my predictions, I saw no suppression of the reproductive or immune measures in the group experiencing high glucose deprivation, yet I did see suppression of reproduction in the group experiencing moderate glucose deprivation. These results were puzzling at first, as I expected that under high levels of glucose deprivation I would see suppression of both reproduction and immunity; however, upon assessment of serum triglyceride levels, I discovered that high levels of glucose deprivation likely resulted in a shift in energy utilization from glucose to free fatty acids. This discovery highlights that the expression of trade-offs is not only dependent on the amount of energy going into the system (i.e., glucose) but is also a product of how and when an animal taps into its energy reserves. In Chapter 3, trade-offs between energetic fuel sources may explain why I observed incomplete attenuation of sickness symptoms in the long-day food-restricted relative to the short-day *ad libitum* group. For instance, even though the short-day *ad libitum* and long-day restricted groups showed similar body masses at the time of LPS treatment, they differed in their body fat levels and circulating leptin concentrations. Consequently, even though these animals shared similar body weights, they showed variation in both their energy sources and signals. The differences in internal energetic environment between these two groups could explain why long-day restricted animals exhibited greater sickness-induced anorexic responses than short-day *ad libitum* animals. Since long-day restricted animals had greater body fat stores than

short-day *ad libitum* animals, then they may have received neuroendocrine signals indicating that energy was not as limited as it was in the short-day animals, even though body mass measurements would suggest otherwise. Thus, when energy reserves are limited, it is possible that trade-offs among different types of energy may influence sickness response intensity.

I identified the presence of a trade-off within the sickness response in Chapter 2, as attenuated LPS-induced hypothermia in the leptin-treated short-day group was coupled with an unpredicted attenuation of LPS-induced anorexia. I had initially predicted that leptin treatment would result in enhanced LPS-induced anorexia in short-day animals if seasonal variation in sickness intensity as a whole was mediated by seasonal changes in leptin levels; however, this attenuation of anorexia suggests that the energetically-costly sickness symptoms may be regulated by different energetic signals. It is possible that attenuating hypothermia resulted in increased energy expenditure and alterations in (unmeasured) energetic signals that facilitated decreased anorexia. Alternatively, in Chapter 4, I observed a potential trade-off across systems as I saw a trade-off between the sickness response and the reproductive system. I predicted that glucose depriving long-day hamsters would result in attenuation of all energetically-expensive sickness symptoms. Instead, I found that glucose deprivation enhanced sickness-induced hypothermia but did not suppress sickness-induced anorexia or body mass loss. Yet, I also found that glucose-deprived hamsters had smaller testes masses, suggesting that energy may have been shunted away from reproductive maintenance to facilitate normal expression of sickness-induced anorexia and body mass loss. From the results of

these four dissertation studies, it is apparent that the processes of energy allocation within and between physiological systems involves precise coordination among systems, fuels, and signals. Shifting our models of energetic trade-offs from simple input-output systems to those that consider additional signaling molecules, physiological processes, and feedback loops will no doubt allow us to gain better understanding of the true mechanisms facilitating energetic allocation among competing systems.

It cannot be ignored that one of the greatest gaps in this body of work is the lack of investigation into the role of the central nervous system in these studies. The unexpected energetic trade-offs I observed in this dissertation likely were facilitated in part by actions to and from the energy-regulating components of the brain. Furthermore, the sickness response is a coordinated set of physiological and *behavioral* responses to infection, and as such, these behaviors are regulated and coordinated by the brain. Thus, the plethora of grey lines pointed toward the brain in Figure 1 are important missing links (and future targets of research) in this body of work. It is likely that the behavioral differences that I observed among LPS-treated groups in each of these final three studies were not mediated directly by changes in energetic signals but rather by changes in the release of and sensitivity to pro-inflammatory and anti-inflammatory cytokines and glucocorticoids. Previous work has shown that Siberian hamsters show seasonal differences in sickness upon treatment with the pro-inflammatory cytokine interleukin (IL)-1β, such that short-day housed hamsters show attenuated sickness in response this treatment as compared to long-day housed hamsters even though they receive

similar quantities of IL-1β (Wen and Prendergast, 2007). Additionally, upon LPS treatment, short-day housed hamsters show lower circulating serum concentrations of IL-6 and lower levels of IL-1β and tumor necrosis factor (TNF)-α from cultured lymphocytes and macrophages, respectively (Bilbo et al., 2002; Prendergast et al., 2003). Much like leptin and insulin do not only affect processes directly related to energy balance, cytokine actions are not limited to immune function, but rather, they can also communicate with and modulate energetic systems and processes (Matarese and La Cava, 2004). Based upon the results of previous explorations of cytokines in Siberian hamsters and the connections between cytokines and metabolism, it seems likely that the energetic treatments I provided the hamsters in this dissertation may have modulated cytokine release and/or sensitivity. Unfortunately, antibodies that recognize and bind to pro-inflammatory cytokines in Siberian hamsters are no longer commercially available, so I was not able to explore these potential connections in this dissertation. However, we hope that in the future, if these antibodies become available, the missing links in Figure 1 will start to appear.

Collectively, the results of this dissertation shed light into the complex relationships among energy sources and signals in the regulation of energetic trade-offs and seasonal sickness response variation. Appropriate coordination of energy use is critical to produce immune responses that will result in effective pathogen clearance. As we move forward in our understanding of the mechanisms that facilitate energy allocation to immunity and other physiological and behavioral processes in variable ecological contexts, future work targeted at cytokines and

neural regulation of these mechanisms will allow us to start to build a complete model of the environmental, hormonal, and neural pathways that allow animals to express optimal immune responses.

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Weil, Z.M., Nelson, R.J., 2012. Neuroendocrine mechanisms of seasonal changes in immune function in: Demas, G.E., Nelson, R.J. (Eds.), Ecoimmunology. Oxford University Press, New York, pp. 297-325.

Wen, J.C., Dhabhar, F.S., Prendergast, B.J., 2007. Pineal-dependent and -independent effects of photoperiod on immune function in Siberian hamsters (Phodopus sungorus). Horm Behav 51, 31-39.

Wen, J.C., Prendergast, B.J., 2007. Photoperiodic regulation of behavioral responsiveness to proinflammatory cytokines. Physiology & behavior 90, 717-725.

Wolowczuk, I., Verwaerde, C., Viltart, O., Delanoye, A., Delacre, M., Pot, B., Grangette, C., 2008. Feeding our immune system: Impact on metabolism. Clin Dev Immunol, 1-19.

Yamamoto, H., Nagai, K., Nakagawa, H., 1984. Bilateral Lesions of the Scn Abolish Lipolytic and Hyperphagic Responses to 2dg. Physiology & behavior 32, 1017-1020.

Zimmerman, L.M., Bowden, R.M., Vogel, L.A., 2014. A vertebrate cytokine primer for eco-immunologists. Funct Ecol 28, 1061-1073.

Zysling, D.A., Demas, G.E., 2007. Metabolic stress suppresses humoral immune function in long-day, but not short-day, Siberian hamsters (Phodopus sungorus). Journal of comparative physiology. B, Biochemical, systemic, and environmental physiology 177, 339-347.

Zysling, D.A., Garst, A.D., Demas, G.E., 2009. Photoperiod and food restriction differentially affect reproductive and immune responses in Siberian hamsters Phodopus sungorus. Funct Ecol 23, 979-988.

Elizabeth D. Carlton

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EDUCATION

2009-2015	PhD, Evolution, Ecology, and Behavior Program, Indiana University, Bloomington, IN
2005-2009	BA, Biology, Kenyon College, Gambier, OH (Graduated *summa cum laude* with highest honors)

RESEARCH EXPERIENCE

2009-2015	**Indiana University Graduate Student**, Advisor: Dr. Gregory Demas, PhD Dissertation Research: Energetic regulation of reproductive-immune trade-offs and seasonal sickness responses in Siberian hamsters.
2008-2009	**Kenyon College Biology Honors Research**, Advisor: Dr. Robert Mauck (Kenyon College), Relationships among parental effort, immune function, oxidative stress, and plumage coloration in Eastern Bluebird adults.
2008	**Kenyon College Summer Science Scholar**, Advisors: Dr. Robert Mauck (Kenyon College); Dr. Kevin Matson (University of Groningen), Growth rates and parental provisioning of lysozyme-supplemented Eastern Bluebird nestlings.
2007	**Kenyon College Summer Science Scholar**, Advisors: Dr. Robert Mauck (Kenyon College); Dr. Mark Haussmann (Bucknell University), Assortative mating by foot color in the Black Guillemot.

FELLOWSHIPS, SCHOLARSHIPS, GRANTS, AND HONORS

2013	NSF Doctoral Dissertation Improvement Grant (Energetic Regulation of Seasonal Sickness Behaviors)
2013	Common Themes in Reproductive Diversity NIH Training Grant Fellowship
2013	Hanna Kolodziejski Fellowship (*honors an Indiana University graduate who excels in research and teaching while striving to improve their community)*
2013	Rowland Mentoring Award (*recognizes Indiana University graduate students for outstanding mentoring of undergraduates)*
2011	Sigma Xi Grant in Aid of Research

2010	National Science Foundation Graduate Research Fellowship
2009	NCAA Walter Byers Postgraduate Scholarship First-Runner Up (*the NCAA's highest academic award, awarded for excellence in academics, athletics, service and potential for success in postgraduate study*)
2009	NCAA Postgraduate Scholarship (*awarded to student athletes who succeed both athletically and academically*)
2008	Barry M. Goldwater Scholarship (*awarded for high academic achievement in undergraduate education and for the potential to pursue successful postgraduate education in science research*)
2008	Elected to Phi Beta Kappa
2008, 2007	Kenyon College Summer Science Scholar (*competitive award providing funding to pursue summer research with a Kenyon College faculty mentor*)

PUBLICATIONS (*Denotes Undergraduate Co-Author)

Carlton, E.D. and Demas, G.E. Glucose and insulin modulate sickness responses in male Siberian hamsters. In review at *General and Comparative Endocrinology*.

Carlton, E.D. and Demas, G.E. 2015. Body mass affects seasonal variation in sickness intensity in a seasonally-breeding rodent. *Journal of Experimental Biology, in press*.

Scotti, M.A., **Carlton, E.D.**, Demas, G.E., and Grippo, A.J. 2015. Social isolation disrupts innate immune responses in both male and female prairie voles and enhances agonistic behavior in female prairie voles (*Microtus ochrogaster*). *Hormones and Behavior* 70: 7-13.

Fasanello, V.J., **Carlton, E.D.**, Pott, M., Marchetto, N.M., Vaughn, E., McGraw, K.J., Mauck, R.A., and Haussmann, M.F. 2015. Monomorphic ornamentation related to oxidative damage and assortative mating in the Black Guillemot (*Cepphus grylle*). *Waterbirds* 38: 106-110.

Demas, G.E. and **Carlton, E.D.** 2015. Ecoimmunology for psychoneuroimmunologists: Considering context in neuroendocrine-immune behavior interactions. *Brain, Behavior and Immunity* 44: 9-16.

Carlton, E.D. and Demas, G.E. 2014. Leptin mediates seasonal variation in some but not all symptoms of sickness in Siberian hamsters. *Hormones and Behavior* 66: 802-811.

Carlton, E.D., Cooper, C.L.*, and Demas, G.E. 2014. Metabolic stressors and signals differentially regulate trade-offs between reproduction and immunity. *General and Comparative Endocrinology* 208: 21-29.

Carlton, E.D., Demas, G.E., and French, S.S. 2012. Leptin, a neuroendocrine mediator of immune responses, inflammation, and sickness behaviors. *Hormones and Behavior* 62: 272-279.

ORAL PRESENTATIONS (*Denotes Presenting Author)

Carlton, E.D.* and Demas, G.E. 2014. Energetic Regulation of Seasonal Sickness Responses in Siberian Hamsters (*Phodopus sungorus*). Center for the Integrative Study of Animal Behavior Annual Conference, Bloomington, IN. Apr. 24-26, 2014

Carlton, E.D.* and Demas, G.E. 2014. Energetic Regulation of Seasonal Sickness Responses in Siberian Hamsters (*Phodopus sungorus*). The Annual Meeting of the Society for Integrative and Comparative Biology. Austin, TX. Jan. 3-7, 2014.

Carlton, E.D.* 2013. In Sickness and In Health: Energetic regulation of seasonal immune responses in Siberian hamsters. Invited seminar for Millikin University Department of Biology, Nov. 1, 2013.

Carlton, E.D.*, Cooper, C.L., G.E. Demas. 2013. Metabolic signals differentially regulate trade-offs between the reproductive and immune systems in female Siberian hamsters. The Annual Meeting of the Society for Integrative and Comparative Biology. San Francisco, CA. Jan. 3-7, 2013.

Carlton, E.D.* 2012. In Sickness and In Health: The role of energetics in seasonal immune responses. Indiana University EEB Brown Bag Series.

POSTER PRESENTATIONS (*Denotes Presenting Author)

Carlton, E.D.* and G.E. Demas. 2013. Energetic regulation of seasonal sickness behaviors in Siberian hamsters (*Phodopus sungorus*). 50th Annual Conference of the Annual Behavior Society. Boulder, CO. July 28-August 1, 2013.

Cooper, C.L.*, **Carlton, E.D.**, and G.E. Demas. 2013. Metabolic signals differentially regulate trade-offs between reproduction and immunity in hamsters. 50th Annual Conference of the Annual Behavior Society. Boulder, CO. July 28-August 1, 2013.

Carlton, E.D.* and G.E. Demas. 2012. Leptin and seasonal variation in sickness responses in Siberian hamsters (*Phodopus sungorus*). Center for the Integrative Study of Animal Behavior Annual Conference, Bloomington, IN. Apr. 5-7, 2012.

Carlton, E.D.* and G.E. Demas. 2012. Leptin and seasonal variation in sickness responses in Siberian hamsters (*Phodopus sungorus*). The Annual Meeting of the Society for Integrative and Comparative Biology. Charleston, SC. Jan. 3-7, 2012.

Tun, K.M.*, Peiris, F.C., **Carlton, E.D.,** Matson, K.D., and R.A. Mauck. 2012. Finding the blue in bluebirds: What does full-spectrum data tell you about individual quality that a camera cannot? The Annual Meeting of the Society for Integrative and Comparative Biology. Charleston, SC. Jan. 3-7, 2012.

Carlton, E.D.*, Matson, K.D., Howard, J.L., Tieleman, B.I., Haussmann, M.F., and R.A. Mauck. 2010. Honest signaling by structurally based plumage coloration in the Eastern Bluebird. Center for the Integrative Study of Animal Behavior Annual Conference, Bloomington, IN. Mar. 25-27, 2010.

Marchetto, N.M.*, **Carlton, E.D.,** Mauck, R.A., and M.F. Haussmann. 2010. Red Hot: Lipid peroxidation and color based assortative mating in black guillemots (*Cepphus grylle*). The Annual Meeting of the Society for Integrative and Comparative Biology. Seattle, WA. Jan. 3-7, 2010.

Matson, K.D., **Carlton, E.D.***, Howard, J.L., Hudak, C.A., Lynn, S.E., and R.A. Mauck. 2009. Effects of experimental immune enhancement, rather than an immune challenge, in a wild bird. The Annual Meeting of the Society for Integrative and Comparative Biology. Boston, MA. Jan. 3-7, 2009.

Carlton, E.D.*, Stewart, S.N.*, Haussmann, M.F., and R.A. Mauck. 2008. Flashy feet: color based assortative mating in the black guillemot (*Cepphus grylle*). The Annual Meeting of the Society for Integrative and Comparative Biology. San Antonio, TX. Jan. 2-6, 2008.

MENTORING EXPERIENCE

2013	Mentor to Chryssa Athans (Indiana University undergraduate) via the Indiana University Women in Science, Math, and Technology Summer Research Program
2012	Mentor to Candace Cooper (Claflin University undergraduate) via the NSF REU Program in Animal Behavior at Indiana University
2011-2012	Mentor to Beth Reinke (Indiana University undergraduate) on immune function aspect of her undergraduate honor's thesis project
2011	Trained Cynthia Downs (University of Nevada-Reno graduate student) in ecoimmunological assays as part of the NSF-funded Research Coordination Network in Ecoimmunology
2010	Mentor to Meelyn Pandit (Indiana University undergraduate) via the NSF REU Program at Mountain Lake Biological Station

2009-2010	Mentor to Amy Sutton (Indiana University undergraduate) on undergraduate honor's thesis project

TEACHING EXPERIENCE

2014	**Instructor**, Ethics and Professional Development course for NSF REU Program in Animal Behavior *(organized and taught a 17 session course in research ethics and professional development skills for the summer REU students)*
2013	**Instructor**, High School Biology, Foundations in Science and Mathematics Summer Program *(taught two week preparatory course for incoming high school biology students; worked with team of biology graduate students to design all course materials)*

Associate Instructor, **Indiana University:**

2010	Introductory Biology Laboratory (L113), Dr. Jim Hengeveld
2010	Biology of Birds (L376), Dr. Susan Hengeveld
2009	Biological Science for Elementary Teachers (Q201), Dr. Pamela Hanratty

Undergraduate Teaching Assistant, Kenyon College:

2007	Introductory Biology Lab (Bio 110), Patricia Heithaus

ACTIVITIES AND SERVICE

2013-2014	Co-organizer of curriculum for Girl's Inc. summer biology camp for teenage girls in the local Bloomington area
2013	Co-Organizer of CISAB Animal Behavior Conference Satellite Symposium (Mechanisms Matter: Hormonal and Neural Regulation in Animal Behavior)
2013, 2012, 2011	Chair of Promotional Committee for CISAB Animal Behavior Conference
2012-2013	Women in Science Living-Learning Community Graduate Student Mentor
2012, 2010	Host for Indiana University Biology Department Graduate Recruitment Weekend
2010-2015	CISAB Animal Behavior Conference Planning Committee Member

AD HOC JOURNAL REVIEWING

Biology Letters, Frontiers in Zoology, Functional Ecology, General and Comparative Endocrinology, Journal of Comparative Physiology-B, Journal of Experimental Biology, Journal of Mammology, Physiology and Behavior, Proceedings of the Royal Society-B